Characterising the role and evolution of temperate bacteriophages in chronic respiratory infections including Cystic Fibrosis and Bronchiectasis

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ABSTRACT

The aim of this research project was to determine whether temperate bacteriophages could be used as markers for bacterial evolution in chronic microbial infections and in the progression of respiratory diseases [Cystic Fibrosis (CF) and Bronchiectasis (BR)]. CF and BR have diverse clinical origins but similar pathophysiological burdens including inflammation and the production of a nutrient rich mucus. This thick, dehydrated mucus is an ideal colonisation site for opportunistic bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*). Some strains of *P. aeruginosa* have been found to harbour multiple inducible temperate bacteriophages that are believed to have an effect on *P. aeruginosa* functionality.

In order to try and answer the research question proposed, the research was split into three subgroups: (1) a cross-infection study utilising 94 clinical *P. aeruginosa* isolates and their associated mixed phage communities to determine changes in phage-host interactions alongside the progression of the clinical disease. We here illustrate that phages induced from the older CF patient isolates were the most infective, whilst the phages originating from the youngest CF patients or the patients with <10 years of BR diagnosis were the least infective.

(2) Metagenome analysis of the total induced viral DNA from each of these 94 *P. aeruginosa* isolates was used to determine whether disease progression offered complexity or additional gene function that would offer a selective advantage for the bacterium or virus in these clinical backgrounds. This research importantly shows differences in phage metagenome complexity and an increase in gene function that correlates with the advancement of disease progression. Therefore, phage metagenomes originating from the older CF patients were the most

enriched in functions relating to survival within the chronic lung. It also shows a snapshot of phage evolution and their impact on the bacteria colonising the lower lung. If higher numbers of phage genes with defined function is a marker of the levels of adaptation and evolution that has occurred, then this research determines that phages isolated from CF metagenomes had undergone more rounds of evolution compared to the BR metagenomes.

(3) Changes in metabolite profiles in the bacterium when infected with a single phage may further show the involvement and subversion of host cell functionality by these phages. A panel of plaque-purified phages were used to create lysogens of lab strain PAO1. Changes in the metabolite profiles between naïve and infected PAO1 were investigated throughout pellicle growth where distinct differences were seen and further show the impact of prophage formation on the core gene function of respiring bacteria. Both the *E. coli* metabolome database and the human metabolome database were utilised in order to identify potential metabolites that were both statistically significant and had a low CV score. We illustrate different metabolite profiles when comparing naïve bacterial strain to lysogen and thus, we establish the intricacy of how the virus subverts host cell functionality. One metabolite was observed that was present in a lysogenic strain and not in the naïve host cell, so potentially leading to the assumption that this metabolite is phage derived. Further LCMS work is required in order to confirm this preliminary finding.

LIST OF CONTENTS

ABBREVIATIONS LIST

Abi	Abortive infection
ADP	Adenosine Diphosphate
AHLs	*N*- Acylhomoserine lactones
AI	Activator proteins
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage fluid
BSA	Bovine Serum Albumin
Bp	Base pair
B. cepacia	*Burkholderia cepacia* complex
BR	Bronchiectasis
cAMP	Cyclic Adenosine Monophosphate
Cas	CRISPR associated protein
CF	Cystic Fibrosis
Cftr	Cystic Fibrosis Transmembrane Regulator gene
CFTR	Cystic Fibrosis Transmembrane Regulator protein
Cfu	Colony forming unit
Cl^-	Chloride ion
COG	Clusters of Orthologous Groups
COPD	Chronic Obstructive Pulmonary Disease
Cos	Cohesive sticky ends
CRISPR	Clustered regularly interspaced palindromic repeats
crRNA	CRISPR RNA
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
CV	Coefficient of variation
Da	Dalton
DNA	Deoxyribose Nucleic Acid
dNMP	deoxyribonucleoside monophosphate
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA

E. coli	*Escherichia coli*
ECM	Extracellular matrix
ECMDB	*E. coli* metabolome database
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EPS	Extrapolysaccharide matrix
ESI	Electrospray ionisation
F	Phenylalanine
g	Gram (s)
GSH	Glutathione
H_2O	Water
HF	High fidelity
HGT	Horizontal Gene Transfer
HPLC	High Performance Liquid Chromatography
HMDB	Human Metabolome Database
HS	High sensitivity
ICTV	International Committee on the Taxonomy of Viruses
ICU	Intensive Care Unit
Kb	Kilobase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
L	Litre
LB	Luria Broth
LC	Liquid Chromatography
LCMS	Liquid Chromatography – Mass Spectroscopy
LES	Liverpool Epidemic Strain
mA	Milliamp
Mb	Mega base pairs
MDR	Multidrug resistant
MG-RAST	Metagenomics Rapid Annotations based on Subsystem Technology
MIC	Minimum Inhibitory Concentration

mL	Millilitre
mm	Millimetre
mM	Millimolar
moi	Multiplicity of infection
mRNA	Messenger RNA
MRSA	Methicillin resistant *Staphylococcal aureus*
MS	Mass Spectroscopy
MTT bromide	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
m/z	Mass to charge ratio
Na^+	Sodium ion
NaOH	Sodium Hydroxide
NFLX	Norfloxacin
NaN_3	Sodium Azide
NGS	Next Generation Sequencing
nm	Nanometre
NMR	Nuclear Magnetic Resonance
NTZ	Nitrofurazone
nut	N utilisation sites
oPLS-DA	Orthologous Partial Least Squares Discriminant Analysis
ORF's	Open reading frames
ori	Origin of replication
P. aeruginosa	*Pseudomonas aeruginosa*
PAM	Protospacer adjacent motifs
P. cepacia	*Pseudomonas cepacia*
PBS	Phosphate-buffered saline
PCoA	Principle Components Analysis
PCR	Polymerase Chain Reaction
PEG8000	Polyethylene Glycol 8000
PFP	Polyhedral, filamentous or pleomorphic morphology
Pfu	Plaque forming unit

PLS-DA	Partial Least Squares Discriminant Analysis
ppGpp	Guanosine pentaphosphate
ppm	Parts per million
PQS	Pseudomonas quinolone signal
QBE	Q binding element
QS	Quorum Sensing
QUT	Q utilisation site
RM	Restriction modification
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per minute
S. aureus	*Staphylococcal aureus*
SCVs	Small Colony Variants
SDS	Sodium Dodecyl Sulphate
SEM	Standard error mean
Sie	Superinfection exclusion
SOD	Superoxide dismutase enzymes
spp	Species
TAE	Tris-acetate-EDTA
ssDNA	Single Stranded DNA
ssRNA	Single Stranded RNA
TA	Toxin Antitoxin system
tracrRNA	*trans* encoded small RNA
tRNA	Transfer Ribonucleic Acid
UPEC	Uropathogenic *E.coli*
UV	Ultraviolet
VAP	Ventilator associated pneumonia
VIP	Variance of Importance plot
VNTR	Variable number tandem repeat
VRE	Vanomycin resistant *Enterococcus* spp
v/v	Volume per volume

W	Tryptophan
WHO	World Health Organisation
w/v	Weight per volume
XTT	2,3-bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
3’	3 prime
5’	5 prime
µL	Microlitres
ϕ	Bacteriophage/phage

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DECLARATION

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted by the School Ethics Committee on 16.10.12.

I declare that the word count of this thesis is 48,365 words (original count).

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Date: 16.09.2016

1. INTRODUCTION

1.1. CHRONIC RESPIRATORY DISEASE

Chronic respiratory diseases affect the airways and structures of the lungs and account for *circa* 4 million deaths annually according to the World Health Organisation (WHO). Approximately 50 % of patients diagnosed with acquired chronic respiratory diseases live in low-to-middle-income countries and it is believed that this acquisition may in part be due to high levels of indoor air pollution and tobacco smoke (WHO, 2004). In higher income countries, both acquired and inherited diseases contribute at equivalent levels to the onset of chronic respiratory diseases. This thesis is focused on an acquired respiratory disease, Bronchiectasis (BR) and an inherited disease, Cystic Fibrosis (CF).

1.2. CYSTIC FIBROSIS

CF is the most common autosomal recessive genetic disorder in Caucasian populations and in 2003, around one in every twenty five children born in the United Kingdom was a carrier of the mutation (Gibson *et al.*, 2003). In Europe (2011), nearly thirty five thousand children and young adults were diagnosed with CF (~ 0.004 %). The prevalence is different in the USA and Canada with the numbers being thirty thousand (~ 0.009 %) and three thousand (~ 0.009 %) respectively (Hoiby, 2011). Population size needs to be considered when drawing conclusions regarding CF prevalence. CF arises due to genetic mutations in the Cystic Fibrosis transmembrane conductance regulator (*CFTR*) gene, a 189 kb gene located on the long arm of chromosome 7. The CFTR gene encodes for a 1480 amino acid protein that is located on the epithelial cell

surface, see figure 1.1. The correct functionality of this protein involves the transportation of negatively charged chloride ions (Cl^-) across the lung epithelia. The CFTR is composed of two domains that span the plasma membrane thus, creating a channel for ion transport. The CFTR is also involved in the maintenance of other epithelial ion transport channels including the positively charged sodium (Na^+) channel. These channels are essential for transport in both the lungs and the pancreas (Frizzell, 1995, Cant *et al.*, 2014). This ion transport controls not only the osmotic potential of the cell but also the essential production of normal free flowing mucus (Riordan *et al.*, 1989, Cant *et al.*, 2014, Frizzell, 1995).

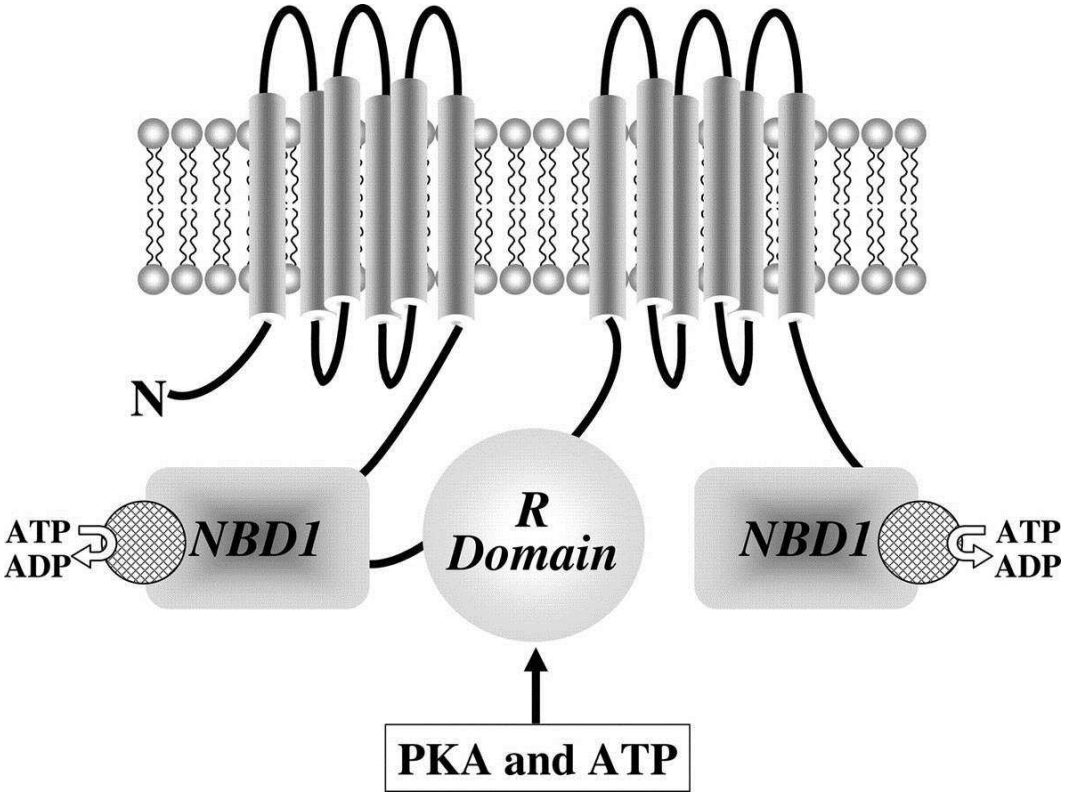


Figure 1.1: Structure of the CFTR protein and details relating to its functionality, from (Goodman and Percy, 2005). Opening and closing the CFTR channel is an energy dependent process. The correct functionality of this channel is required for the transportation of Cl^- ions across the epithelial membrane, in order to ensure that the correct mucus levels are retained within the airways and around the body. In order to generate the energy for this transportation, the regulatory domain (R domain), requires phosphorylation which in turn allows for adenine triphosphate (ATP) hydrolysis to occur (Riordan *et al.*, 1989). ATP hydrolysis occurs at the nucleotide binding domains (NBD1 and NBD2) and the production of free phosphate ions and adenosine diphosphate (ADP) releases the energy required to ensure that the channel opens and closes effectively (Hwang and Sheppard, 2009). The activity of this channel is also dependent on the presence of intracellular ATP (Sheppard and Welsh, 1999, Berger *et al.*, 1991). When the R domain is not phosphorylated, the channel is closed which causes the movement of Cl^- to cease. Phosphorylation of R and the subsequent binding of the ATP onto the nucleotide binding domains occurs in a sequential manner, which increases the likelihood of the channel remaining open (Goodman and Percy, 2005).

1.3. BRONCHIECTASIS

BR is the irreversible dilation of the lower bronchi caused by chronic airway inflammation. Subsequent scarring of the lung tissue through reactive oxygen species generation or lung obstruction transcends into consistent and recurrent bacterial chest infections (Boyton, 2012, Cole, 1985, Barker, 2002, Laennec, 1962). BR doesn't have a clear cause as it can be triggered by a chronic infection or it can be the downstream problem of a localised lung trauma event. Around half of the known causes of BR are idiopathic, table 1.1 details the proposed causes for the non-idiopathic BR patients.

Upon reduction, reactive oxygen species are formed and these can have detrimental effects on lung health and functionality (Anderson, 1996). Reactive oxygen species aside from having a potentially damaging role in the human body are also involved in numerous other important cellular events such as protein phosphorylation, transcription factor activation, gene expression, DNA synthesis and cell proliferation (Hoidal, 2001, Schreck *et al.*, 1991, Sen and Packer, 1996). Reactive oxygen species are known to also have a role in phagocytosis so these species can protect the host from the effects of pathogenic organisms. Reactive oxygen species induce polymorphonuclear leukocytes which in turn triggers phagocytosis. Reactive oxygen species can be effectively cleared from a healthy human body. For example, Superoxide Dismutase (SOD) can convert superoxides into hydrogen peroxide in the mitochondria and this hydrogen peroxide is removed from the body by catalases (Anderson, 1996, Chance *et al.*, 1979). Another important antioxidant that is present within mammalian cells is Glutathione (GSH), this has been shown to have a critical role in preventing lung epithelial cell apoptosis (Hoidal, 2001). It has been shown that the GSH level is around 100 fold greater in the alveolar epithelial lining fluid compared to the plasma (van der Vliet *et al.*, 1999). However, in numerous inflammatory

conditions including CF/BR, GSH levels are reduced and this reduction is potentially a contributing factor to the elevation observed in epithelial cell apoptosis (Roum *et al.*, 1993, Hoidal, 2001).

Apoptosis therefore, may play a role in the scarring observed in BR lungs. Due to a lack of mucociliary clearance, a vicious cycle develops where persistent bacterial colonisation of the mucus leads to chronic bronchial mucosal inflammation and progressive tissue destruction (Rogers *et al.*, 2013). This inflammation can then drive further bacterial infections, which in turn drives further tissue damage and inflammation. This cycle leads to the typical symptoms observed in BR patients such as chronic and frequently purulent expectoration, multiple exacerbations and progressive dyspnoea (Rogers *et al.*, 2013). Exacerbations are when the disease symptoms become more serve, normally these last for a couple of days and they are normally triggered by either an environmental stimulus or a microbial/viral infection.

Table 1.1: Diseases that can contribute to the onset of BR, adapted from (Boyton, 2012). Recognised causes of bronchiectasis; around 50 % of the diagnosed cases of BR are idiopathic but the remaining 50 % of cases are often associated with other clinical conditions with BR being a disease endpoint.

Cause	Example	Reference
Post infection	Can be caused by bacterial and viral infections e.g. Measles's	(Boyton, 2012)
Mucociliary clearance defects	Primary ciliary dyskinesia	(Morillas *et al.*, 2007)
Inhalation of toxins	Toxic gases Smoke inhalation	(Boyton, 2012)
Lung obstruction	Foreign Body	(Boyton, 2012)
Immunodeficiency	Hypogammaglobulinemia - characterised by reduction of all types of gamma globulins	(Kainulainen *et al.*, 1999)
Malignancy	Cancer and Leukaemia Chemotherapy (related to treatment)	(Boyton, 2012)
Autoimmune conditions	Rheumatoid Arthritis – related to auto antibodies and abnormal serum immunoglobulin levels & Systemic lupus erythematous – causes systemic inflammation in the heart, joints, skin, lungs, blood vessels, liver, kidneys	(McMahon *et al.*, 1993, Cohen and Sahn, 1999) (Higenbottam *et al.*, 1980)

	and the nervous system & Relapsing polychondritis – damage to cartilage containing tissues, it can become life threatening when starts to affect the respiratory tract & Ulcerative colitis – an inflammatory bowel disease that mainly affects the colon	(Cohen and Sahn, 1999)
Congenital conditions	Tracheobronchomegaly – abnormal widening of the upper airways leading to lower airway infections, also known as Mounier-Kuhn syndrome	(Schwartz and Rossoff, 1994)
Other	Yellow nail syndrome Mercury poisoning Fibrosis of the lung for example, Sarcoidosis	(Cohen and Sahn, 1999)

1.4. THE INCDENCE OF BACTERIAL SPECIES COLONISING THE CHRONIC LUNG

The thick, nutrient mucus lining the lungs of chronic lung disease patients provides an environment that is ideal for opportunistic bacteria to colonise and proliferate within. Opportunistic bacteria are classed as species that can take advantage of environmental changes and rapidly adapt to successfully colonise new niches (Peloux, 1985).

In 2010, the commonly isolated bacterial species of the CF lung were reviewed and it was seen that they included *Staphylococcus aureus (S. aureus)*, *Haemophilus influenzae* (*H. influenzae*), *Stenotrophomonas maltophilia, Achromobacter xylosoxidans, Burkholderia cepacia* complex C and *Pseudomonas aeruginosa* (*P. aeruginosa*) (LiPuma, 2010).

It has been proposed that the pattern of bacterial colonisation in paediatric CF patients usually involves early colonisation with *S. aureus* and non – capsulated *H. influenzae* (Hart and Winstanley, 2002). These pathogens are the main 'early' opportunistic bacterial species to colonise the lungs of paediatric CF patients. They remain the main colonisers until their succession by *P. aeruginosa* and *Burkholderia cepacia* complex C. This succession is believed to occur around school age or during early adolescence (Tümmler and Kiewitz, 1999). *Burkholderia cepacia* complex C is only found in a small percentage of CF patients. It is a metabolically versatile bacterium that is found in a wide variety of environmental niches and its association with the CF lung can have serious health implications, somewhat more so than *P. aeruginosa* (Davis *et al*., 1996, Govan and Deretic, 1996).

Recent studies involving metagenomics and 16S rRNA (ribosomal RNA) gene sequencing have highlighted that the complexity of the microbial communities in

the CF and BR lung is greater than previously perceived. However, care must be taken when using the data generated via 16S rRNA sequencing as the data can sometimes make it hard to resolve taxonomies at the species level and may also introduce bias via the PCR amplification step in the sequencing programme (Lim *et al.*, 2014, Filkins *et al.*, 2012, Cai *et al.*, 2013). *P. aeruginosa* growth in the mucus lining the chronic lung generates steep oxygen gradients which allows for the colonisation of the lung with anaerobes thus, making chronic lung infections polymicrobial in nature (Tunney *et al.*, 2008). It has been seen that anaerobes are present in comparable numbers to *P. aeruginosa* and *S. aureus* (Worlitzsch et al., 2009). *Staphylococcus saccharolyticus* and *Peptostreptococcus prevotii* are the most prevalent anaerobes detected in the CF lung (Worlitzsch *et al.*, 2009). The presence of *P. aeruginosa* doesn't have any impact on the likelihood of the onset of colonisation by anaerobes (Worlitzsch *et al.*, 2009).

Filamentous fungi have also been isolated from the respiratory tract of CF patients, in particularly *Aspergillus fumigatus* which is associated with reduced lung function (Saunders *et al.*, 2016). Fungi comprise under < 0.1 % of the total microbiota of the lung but they have been shown to have major roles in maintaining structure, metabolic function and immune priming functions (Zelante *et al.*, 2016). These studies have emphasised the complexity of the microbiota of the lower lung in chronic respiratory disease patients, the viral diversity is described in detail in section 1.4.1.

The bacterial species which colonise the BR lung are similar to the bacterial species isolated from the CF lung but there are a few exceptions. Bacterial isolates that are present in both disorders include *P. aeruginosa*, *H. influenzae* and *S. aureus*. Bacterial species that are more descriptive of the BR lung include *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Rogers *et al.*, 2013, Pasteur *et al.*, 2000, Nicotra *et al.*, 1995, Pang *et al.*, 1989, Angrill *et al.*, 2002,

Barker, 2002). Emerging evidence has led to the observation that *P. aeruginosa* and *H. influenzae* colonisation of the BR lung stimulates numerous neutrophilic and inflammatory responses, which may be a driving force for the numerous cycles of bacterial infections which are characteristic of BR (Barker, 2002, Angrill *et al.*, 2001). Numerous research groups have shown that *P. aeruginosa* colonisation within the BR lung correlates to increased sputum production, more hospitalisations and reduced quality of life (Wilson *et al.*, 1997, Ho *et al.*, 1998, Barker, 2002).

1.4.1. Viral diversity within the chronic lung

Bacterial communities in the CF lungs are spatially heterogeneous, so it has been proposed that the viral communities in the lower lung may also vary spatially leading to localised viral infections (Willner *et al.*, 2012). Spatial heterogeneity is the uneven distribution of various concentrations of the different species within a microenvironment. It has been seen that the apical lobes of the lungs harbour very few viral genotypes, this niche in 2012 was proposed to be one of the least inhabited niches known (Willner *et al.*, 2012). The basal lobes harbour ~ 10^2 viral genotypes and so these distribution differences show the spatial nature of viral species within the lungs (Willner *et al.*, 2012). Generally, bacteriophages (section 1.6) outnumber eukaryotic viruses both in abundance and diversity in the human virome which includes the lungs (James *et al.*, 2015, Lim *et al.*, 2014). Metagenomic analysis of the CF lung virome couldn't identify all the viral species present but out of the proportion identified it was seen that the majority were mostly infective against CF pathogens and these included many bacteriophages which targeted *Pseudomonas* species (Lim *et al.*, 2014, James *et al.*, 2015).

Before the invention of polymerase chain reaction (PCR), the incidence of viral infections in the CF lung was believed to be low (around 10 - 28 % of patients) (Wang *et al.*, 1984, Ong *et al.*, 1989, Smyth *et al.*, 1995, Ramsey *et al.*, 1989, Singanayagam *et al.*, 2012). However, since the advent of this technology the detection of viruses in CF patients has risen to around 50 – 60 % (Armstrong *et al.*, 1998, Wat *et al.*, 2008, ALMEIDA *et al.*, 2010, Singanayagam *et al.*, 2012).

There is now an increasing amount of evidence which states that viral infections particularly the influenza virus and the respiratory syncytial virus increase the incidence and severity of secondary bacterial infections such as sepsis and pneumonia (Bustamante-Calvillo *et al.*, 2001, Kim *et al.*, 1996, KORPPI *et al.*, 1989, Schwarzmann *et al.*, 1971, Avadhanula *et al.*, 2006). It is proposed that viral infections may predispose the sufferer to develop secondary bacterial infections due to impaired mucocillary function, host inflammation alterations and also by damaging the respiratory epithelium (Murphy and Sethi, 1992, Wilson and Cole, 1988, Avadhanula *et al.*, 2006). The most frequently isolated bacterial species from the chronic lung environment which have associated viral infections are non-typeable *H. influenzae* and *Streptococcus pneumonia* (Smith *et al.*, 1976, White *et al.*, 2003, Avadhanula *et al.*, 2006). In 1996, it was observed that 85 % of new pseudomonal colonisations of the chronic lung were preceded by an upper respiratory viral infection (Collinson *et al.*, 1996, van Ewijk *et al.*, 2005).

Viral infections in CF have been associated with an increase in patient morbidity as infections result in prolonged hospital stays, a persistent but noticeable decrease in pulmonary function, increased antibiotic use and a higher occurrence exacerbation events (van Ewijk *et al.*, 2005). The respiratory syncytical virus is found in the highest numbers in CF patients, it is found in some cohorts in 9 – 58 % of virus infected CF patients (Abman *et al.*, 1988, Armstrong *et al.*, 1998, van Ewijk *et al.*, 2005). This is followed by Influenza virus (type A and B) which has

been reported in 12 – 27 % of CF patients infected with a respiratory virus (Hordvik *et al.*, 1989, van Ewijk *et al.*, 2005). Other viral entities have been detected but their detection rate is low; Parainfluenza, Enteroviruses, Epstein Barr virus and cytomegalovirus (Pribble *et al.*, 1990, van Ewijk *et al.*, 2005).

1.5. *PSEUDOMONAS AERUGINOSA*

P. aeruginosa is a member of the γ-subdivision of the *Proteobacteria* and is a species of the *Pseudomonas* genus. *P. aeruginosa* is normally between 1 to 5 µM long and 0.5 to 1.0 µM in diameter. *P. aeruginosa* harbours a GC rich (65 %), double-stranded DNA chromosome (dsDNA) with the chromosome ranging in size from between 5.2 to 7 Megabase pairs (Mb) (Tümmler and Kiewitz, 1999, Qiu *et al.*, 2006). *P. aeruginosa* is a Gram negative bacterium with a highly conserved genetic core region and a mosaic accessory element. This accessory element is generated due to the presence of multiple genomic islands (Winstanley *et al.*, 2009). *P. aeruginosa* was originally isolated from the soil as a plant pathogen where it was found to be capable of breaking down polycyclic aromatic hydrocarbons, in order to generate rhamnolipids and quinolones (Maier and Soberon-Chavez, 2000, Rendell *et al.*, 1990). In accordance with the previously described opportunism of *P. aeruginosa*; *P. aeruginosa* has now been isolated from a range of human clinical disorders such as urinary tract infections (Salmen *et al.*, 1983, Ferroni *et al.*, 1998), burn victims (Japoni *et al.*, 2009, Church *et al.*, 2006, Altoparlak *et al.*, 2004, Estahbanati *et al.*, 2002), and in CF, BR and Keratitis patients (Dart and Seal, 1988, Stewart *et al.*, 2011). Nosocomial pneumonia in patients who require mechanical ventilation is one of the most common hospital infections and *P. aeruginosa* often plays a key role in the onset of disease in this patient subgroup (Bodey *et al.*, 1983, Vallet *et al.*, 2001). The isolation of *P. aeruginosa* from the hospital environment compounds the notion

that *P. aeruginosa* infections have serious implications in human health and this supports work undertaken by researchers who want to determine how to control the spread of *P. aeruginosa* infection within an environment.

In order for *P. aeruginosa* to thrive in so many environmental niches, it is not surprising that the bacterium utilises multiple respiratory pathways which relate to the environment in which the bacterium is residing. *P. aeruginosa* is traditionally thought of as an aerobic bacterium with a coccobacillus phenotype but it can also be referred to as a facultative anaerobe. Quinn *et al* (2014) studied the sputum of CF patients using Kyoto Encyclopaedia of Genes and Genomes (KEGG) profiles and it was seen that nitrate reductase was an abundant electron acceptor. This indicates the role that *P. aeruginosa* has in nitrogen denitrification in the CF lung and therefore, in anaerobic metabolism (Quinn *et al.*, 2014). This was also emphasised as the concentration of ammonia in the sputum was elevated in relation to the saliva of healthy individuals, so again showing the ability of *P. aeruginosa* to persist in anaerobic conditions (Quinn *et al.*, 2014). It is also possible that this nitrogen by-product will have an effect on the pH of the mucus lining the airways, which in turn may influence which other bacteria can co-colonise this particular niche. Another elevated compound observed is Cytochrome C oxidase. This finding further supports the hypothesis that *P. aeruginosa* can respire and survive in anaerobic conditions, such as the biofilms lining the airways of patients (Quinn *et al.*, 2014).

1.5.1. Phenotypic differences between *P. aeruginosa* strains isolated from chronic respiratory disease patients

It has been reported that CF patients are initially colonised with a clonal population of *P. aeruginosa* (Martin *et al.*, 1995, Oliver *et al.*, 2000). Therefore,

studies that show variation in the behaviour or phenotype of *P. aeruginosa* during the course of an infection are of particular interest. This again becomes increasingly interesting if the core genetic backbone shows no alteration throughout the infection timeline. In a bacterial population, clones are defined as bacteria that are not distinguishable by genetic methods and have descended from a common ancestor (Spratt, 2004, Maatallah *et al.*, 2011). Several studies have recently proposed that *P. aeruginosa* doesn't have a clonal population like originally thought, rather *P. aeruginosa* has a non-clonal population which is dotted with highly successful epidemic clones (Maatallah *et al.*, 2011, Denamur *et al.*, 1993, Picard *et al.*, 1994, Cornelis, 2008, Curran *et al.*, 2004, Kiewitz and Tümmler, 2000, Scott and Pitt, 2004).

Transmissible strains have been isolated which can infect a large number of patients and these strains have been seen to move around human host populations. These strains also exhibit phenotypic differences when compared to lab strains of *P. aeruginosa* and so these differences may allude to the success rates of these transmissible strains. Examples of some of these transmissible strains are described in table 1.2 along with the pre-mentioned phenotypic differences.

Interestingly, clinical studies have often showed that clonal populations of *P. aeruginosa* in fact can exhibit diverse colony morphologies (Drenkard and Ausubel, 2002b, von Gotz *et al.*, 2004, Haussler *et al.*, 2003, Workentine *et al.*, 2013). When investigating colony morphologies between clonal isolates, differences are proposed when there are alterations relating to motility (Lee *et al.*, 2005a, Leone *et al.*, 2008, Workentine *et al.*, 2013), quorum sensing (Workentine *et al.*, 2013, Wilder *et al.*, 2009, Lee *et al.*, 2005a), production of virulence factors (Lee *et al.*, 2005a, Leone *et al.*, 2008, Fothergill *et al.*, 2010, Workentine *et al.*, 2013), antibiotic susceptibility (Workentine *et al.*, 2013, Foweraker *et al.*, 2005,

Foweraker *et al.*, 2009, Fothergill *et al.*, 2010) and biofilm formation (Lee *et al.*, 2005a, Workentine *et al.*, 2013). Some key virulence factors of *P. aeruginosa* are listed in table 1.3, the overexpression or down-regulation of some of these factors may trigger the phenotypic differences that are observed within *P. aeruginosa* populations.

During the course of *P. aeruginosa* infection within the lower airways of the CF/BR lung, *P. aeruginosa* can alter its phenotype but these colony phenotypic alterations are not always due to genetic changes occurring within the core *P. aeruginosa* genome (Zierdt and Schmidt, 1964, Häußler *et al.*, 1999). These phenotypic alterations include the production of a mucoid phenotype (Deretic *et al.*, 1994), formation of dwarf/small colony variants (Martin *et al.*, 1995), generation of lipopolysaccharide deficient variants (Dasgupta *et al.*, 1994), formation of a rough phenotype (Martin *et al.*, 1995), and some *P. aeruginosa* isolates have also been observed to have a hyperpilated phenotype upon isolation from the chronic lung (Deziel *et al.*, 2001). All these phenotypic alterations enforce the heterogenic nature of the bacterial communities which colonise the CF/BR lung.

Table 1.2: Examples of some of the transmissible strains of *P. aeruginosa* isolated from CF patients around the globe. Some phenotypic differences between the various strains has been alluded to which may explain the success rates of these transmissible strains globally.

***P. aeruginosa* transmissible strain**	**Phenotypic differences**	**References**
Liverpool (LES)	Greater virulence observed, Can replace previously established *P. aeruginosa* strains, Infect non-CF parents/carers, Possess genomic islands, Upregulation of antimicrobial sensitivity genes, Non-motile due to the lack of a visible flagella/pili, Premature expression of Quorum sensing genes	(Scott and Pitt, 2004, McCallum *et al.*, 2001, McCallum *et al.*, 2002, Parsons *et al.*, 2002, Winstanley *et al.*, 2009, Salunkhe *et al.*, 2005)
Manchester (MES)	Identified in 2001 in a Manchester (UK) CF clinic, Highly resistant to anti-pseudomonal antibiotics,	(Scott and Pitt, 2004, Jones *et al.*, 2001, Fothergill *et al.*, 2012)

	Contains only one pathogenicity island – MA island	
Australian (AES-1/AES-2)	AES-1 first identified in Melbourne, now also isolated in Brisbane AES-1 causes increased hospital visits, AES-2 is more common in Brisbane, AES-2 exhibits elevated antibiotic resistance, Both strains cause an increase in virulence factors such as protease IV, elastase and alkaline protease, Both strains also exhibit up-regulation of type III secretion systems and enhanced biofilm growth	(Scott and Pitt, 2004, Armstrong *et al.*, 2002, Syrmis *et al.*, 2004, O'Carroll *et al.*, 2004, Smith *et al.*, 2006, Tingpej *et al.*, 2007, Manos *et al.*, 2008, Manos *et al.*, 2009, Fothergill *et al.*, 2012)
Clone C	Widespread distribution in both clinical and environmental settings, Not detected to cause an elevation in biofilm formation or antibiotic	(Scott and Pitt, 2004, Römling *et al.*, 2005, Fothergill *et al.*, 2012)

	resistance	
Midlands-1	Little is known about this strain even though it has a high prevalence within the CF community, Not highly antibiotic resistant, No link to increased morbidity, Possible link between age and the time of acquisition of the strain	(Scott and Pitt, 2004, Chambers *et al.*, 2005, Fothergill *et al.*, 2012)
Stoke/Trent	Little is known about this strain	(Fothergill *et al.*, 2012, Scott and Pitt, 2004)
Sheffield	Little is known about this strain	(Fothergill *et al.*, 2012, Edenborough *et al.*, 2004)
Leeds	Little is known about this strain	(Fothergill *et al.*, 2012, Denton *et al.*, 2002)

Table 1.3: Some key virulence factors of *P. aeruginosa.* Through the overexpression or down-regulation of some of these factors it is proposed that some of they will generate some of the phenotypic differences observed within *P. aeruginosa* populations.

Virulence factor	Role of this factor in *P. aeruginosa*	Reference
Quorum Sensing (QS)	High bacterial cell density triggers the QS system, the activation of this system leads to the production of two *P. aeruginosa* toxins: Elastase Pyocyanin	(Hauser, 2011)
Type II Secretion system	System utilised by bacteria in order to inject a set of toxins into the cytosol of the host, In *P. aeruginosa* the toxins injected are: ExoU ExoS both of the toxins have links to bacterial virulence	(Hauser, 2011, Hauser, 2009)
Lipopolysaccharide O-antigen	Variable polysaccharide that decorates the outer surface of *P. aeruginosa*	(Hauser, 2011, Pier, 2007)

	and protects the bacterial cell from complement mediated lysis, Differences in the O-antigen of *P. aeruginosa* also allows for bacterial serotyping	
Flagella and pili	These bacterial appendages are required for adhesion, motility and mediating an inflammatory response in the host's immune system	(Gellatly and Hancock, 2013)
Proteases	Bacterial proteases can degrade immunoglobins and fibrin in order to disrupt the functioning of the tight epithelial junctions of the host's immune system, In the lung proteases can degrade the lung surfactant which allows for enhanced bacterial colonisation, Some examples of *P.*	(Gellatly and Hancock, 2013, Kipnis *et al.*, 2006, Fleiszig and Evans, 2002, Laarman *et al.*, 2012, Toder *et al.*, 1994, De Kievit and Iglewski, 2000)

	aeruginosa proteases include: Alkaline protease LasA LasB Protease IV	
Exotoxin A	Inhibits the host's elongation factor 2 which affects protein synthesis, which in turn causes death of the host cell	(Gellatly and Hancock, 2013)
Lipases and phospholipases	These products break down surfactant lipids and phospholipids of host cell membrane's in order to allow the bacterial strains to pass unaided into their host cell	(Gellatly and Hancock, 2013, Kipnis *et al.*, 2006)

1.5.1.1. Small colony variants

Small colony variants (SCVs) are often resistant to aminoglycosides and have been seen to develop in *P. aeruginosa* populations both *in vivo* and *in vitro*. Drenkard and Ausubel (2002) indicated that *P. aeruginosa* was capable of undergoing transient phenotypic changes and that these changes allowed for the appearance of antibiotic resistant SCVs both *in vitro* and *in vivo.* This conversion is a result of phase variation; a common phenomenon in Gram negative bacteria that is governed by alterations in environmental signals resulting in an change to the observable phenotype of the bacterial cell (Drenkard and Ausubel, 2002b, Henderson *et al.*, 1999). A unique feature of *P. aeruginosa* SCVs is their ability to revert back to their wild-type, fast growing phenotypes after the pressure of antibiotic treatment has been reduced and the surrounding environment has become more stable and less stressed due to the antibiotic treatment (Häußler *et al.*, 1999). This phenotype is often selected for when *P. aeruginosa* has been subjected to prolonged antibiotic therapy and is growing as part of a biofilm (Deziel *et al.*, 2001, Häußler, 2004). *P. aeruginosa* biofilms can also harbour multiple *P. aeruginosa* isolates which all have different phenotypes thus, indicating the diversity and synergy of *P. aeruginosa* (Drenkard and Ausubel, 2002b, Woo *et al.*, 2012).

The detection of SCVs in the chronic CF lung has been not only been associated with lesser lung function but SCV-positive patients are also normally lower in weight when compared to their CF counterparts who are the same height but SCV-negative (Häußler *et al.*, 1999). SCV-positive patients are given anti-psuedomonal drugs via aerosols because it increases the concentration of the drug that can reach the infected lower lung. Even though these aerosolised drugs have been seen to decrease the density of *P. aeruginosa* in the chronic CF lung, they do not eradicate SCVs completely (Häußler *et al.*, 1999, Ramsey *et al.*,

1993). SCV formation is a key adaptation technique for *P. aeruginosa* and it may explain why this bacterium can become so predominant in chronic lower lung infections (Malone *et al.*, 2012).

1.5.1.2. Mucoid phenotype

The mucoid phenotype of *P. aeruginosa*, figure 1.2, is commonly isolated in chronic respiratory disease patients and it is caused by the overexpression of alginate, normally through a mutation in the *mucA* transcriptional regulator gene (Lee *et al.*, 2005a, Speert *et al.*, 1990, Mathee *et al.*, 1999, Govan and Deretic, 1996). The appearance of a mucoid *P. aeruginosa* phenotype is associated with a poorer prognosis and a deterioration in lung function, due to the increased tissue damage which is caused by the overproduction of alginate (Høiby *et al.*, 2001). Alginate overproduction causes this deterioration in lung function through accelerating biofilm production which allows for cells to become antibiotic resistant. Alginate also causes cells to become resistant to opsonisation and so this affects the host's immune system. Another alteration that occurs in the host's immune system is a decrease in polymorphonuclear leukocyte chemotaxis. All these factors trigger the reduction in lung function observed in CF/BR patients however, alginate overproduction can also be advantageous for *P. aeruginosa* cells colonising the lungs.

Alginate as mentioned allows for the formation of an extracellular matrix (ECM) which encases the bacterial cells and so provides them with protection from external challenges like antibiotics and antimicrobials for example (Damron *et al.*, 2011). It is believed that the optimum time to treat chronic CF lung infections is before conversion to the mucoid phenotype, as the bacterial infection subsequently becomes increasingly difficult to treat with antimicrobials and

antibiotics due to the ECM (Damron and Goldberg, 2012). Alginate can therefore, be referred to as a virulence factor that *P. aeruginosa* can utilise in order to successfully colonise the chronic lung. However, non-mucoid phenotypes of *P. aeruginosa* can be isolated after long-term colonisation in the CF lung alongside mucoid *P. aeruginosa* isolates (Mahenthiralingam *et al.*, 1994). This observation implies that the mucoid phenotype is not essential for long-term adaptation and colonisation of the chronic lung. Alginate is a polymer composed of D-mannuronic and L-guluronic acid (Hogardt and Heesemann, 2010) and its production is tightly controlled by the *algD-algA* operon. This operon is controlled by AgtT (AlgU) which controls the expression levels of *algD*. *algT* belongs to the operon which also includes *mucA,* which as described previously has a role in mucoid phenotype conversion.

It is of particular interest as to how one mutation can have such dramatic effects on the phenotype of the bacterium and how the bacterial cell evolves, in order to overcome the host's defence system and aid its own survival within the chronic lung (Hogardt and Heesemann, 2010, Govan and Deretic, 1996).

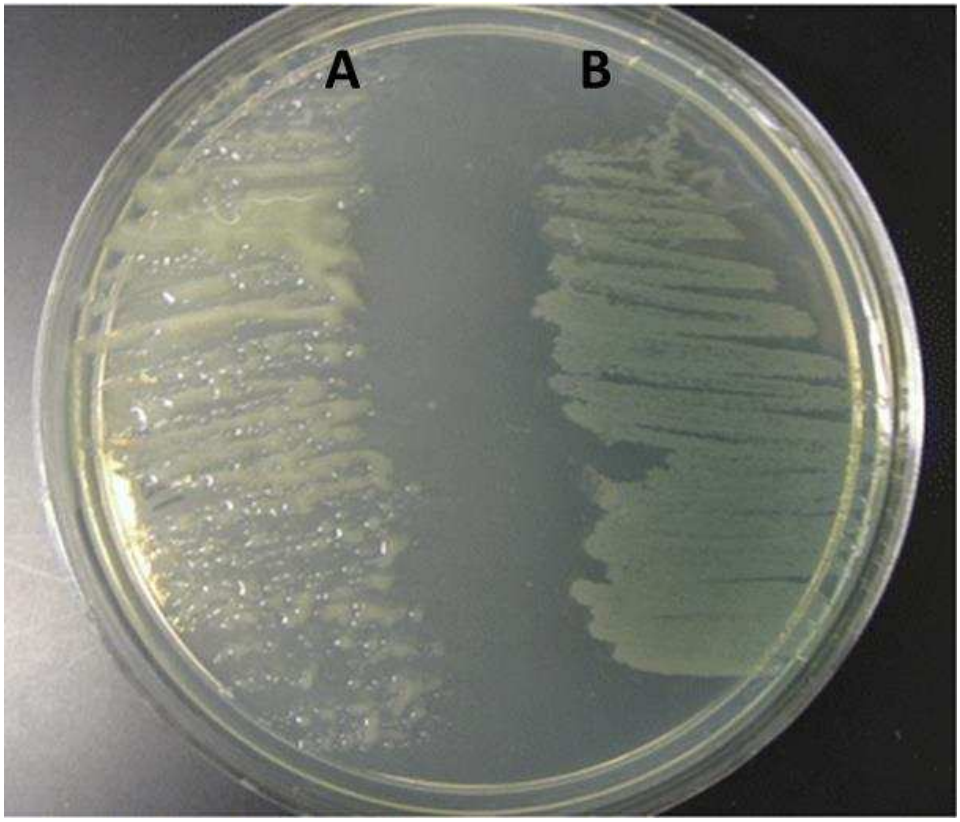


Figure 1.2: Phenotypic differences that can be observed between Pa isolates originating from the chronic lung (Hauser *et al*., 2011). The two different phenotypes are shown on this image, mucoid (A) and non-mucoid (B). Both phenotypes are often isolated side-by-side from chronic respiratory disease patients.

1.5.2. *P. aeruginosa* colonisation of the chronic lung

When CFTR is functioning correctly, long term colonisation of *P. aeruginosa* within the mucus layer doesn't occur as the *P. aeruginosa* is detected and internalised. When a patient harbours a faulty CFTR, not only can *P. aeruginosa* remain associated with the airway due to impaired binding to the mucus layer but the concentration of sialylated glycolipids present on the surface of mucins is also higher than normal and these glycolipids provide additional binding receptors for *P. aeruginosa* (Callaghan and McClean, 2012). Another alteration that occurs in CF lungs is an increase in the number of fucosyl residues present on membrane glycopeptides to which *P. aeruginosa* can bind via fucose specific lectins. So this alteration further increases the amount of binding sites available for *P. aeruginosa* within the chronic lung environment (Callaghan and McClean, 2012). Cell death is elevated in CF airways due to raised ceramide levels and the associated cell debris generates more adherence sites for *P. aeruginosa*.

P. aeruginosa is also able to interact with the host's immune system, for example, through the overproduction of alginate (section 1.5.1.2) which allows for the bacterium to become more successful when colonising the chronic lung. A common problem detected in CF patients is reduced macrophage clearance. The amount of white blood cells that are able to engulf and phagocytise bacterial pathogens is reduced which gives *P. aeruginosa* a greater chance of avoiding host cell detection and enabling its survival in the chronic CF lung (Callaghan and McClean, 2012).

Comparative genomic studies have revealed that bacteriophages also play key roles in the evolution and adaptation of *P. aeruginosa* in the chronic lung as well as influencing the genomic diversification of the bacterial population (Davies *et al.*, 2016b, Brüssow *et al.*, 2004). Bacteriophages are described in more detail in section 1.6, as well as in further chapters where the interactions between the

bacterial and phage communities within the CF/BR lungs have been studied in greater depth (Chapters 3 and 4 predominantly). Bacteriophages in brief, may allow *P. aeruginosa* to adapt to the chronic lung as they can help the bacteria to develop a biofilm community which enhances the antimicrobial resistance profiles of the bacterium whilst also enhancing the ability of the bacterium to avoid host cell defence systems (Silby *et al.*, 2011, Costerton *et al.*, 1995, Drenkard and Ausubel, 2002b). It has previously been mentioned how virulence factors of *P. aeruginosa* (section 1.5.1) can also lead to the development of a biofilm community so showing that *P. aeruginosa* colonisation to the CF/BR lung is multifactorial.

Biofilms/pellicles are described in more depth later in this thesis (chapter 5). However, biofilms occur when cell-density reaches a threshold level and this triggers the activation of the quorum sensing system. Upon activation of this system, an extracellular matrix is generated which encases the bacteria cells and provides them with protection from antimicrobials, antibiotics and detergents. A pellicle forms by the same principles as a biofilm however, their production requires an air-liquid interface. Pellicles are studied in this thesis as it is believed that they are more representative of the chronic lung environment.

The CF airways are a complex network of different niches and these areas allow for the development of bacterial populations which are well adapted to colonise the particular niches due to the individual selection pressures imposed upon them by the different areas of the lung (Marvig *et al.*, 2015). The heterogeneity of the lung has been previously mentioned when the various viral species colonising the lung where discussed (section 1.4.1).

1.5.3. Identification and characterisation of *P. aeruginosa* strains in CF/BR

P. aeruginosa isolates collected at routinely attended CF clinics are typed via variable number tandem report (VNTR) in order to aid strain identification (Turton *et al.*, 2010). VNTR typing involves determining the number of repeats at nine variable-number tandem repeat sites throughout the genome. These repeats are targeted using Polymerase Chain Reaction (PCR) which allows for the rapid identification of bacteria and this has large implications in clinical settings (Turton *et al.*, 2010). The most prevalent *P. aeruginosa* strain isolated in a study of adult CF patients being treated at CF clinics throughout England and Wales was the Liverpool Epidemic Strain (LES), followed by the Manchester Epidemic Strain, Midlands 1, Melbourne Strain and Clone C (Fothergill *et al.*, 2012, Scott and Pitt, 2004). Various other strains of *P. aeruginosa* were isolated but these were classed as unique until they had been identified in a larger patient cohort (Fothergill *et al.*, 2012, Scott and Pitt, 2004). These transmissible strains have been previously described in table 1.2.

LES was initially isolated in 1996 due to its high incidence rate and also due to its resistance to β lactamase antibiotics (Cheng *et al.*, 1996). LES has been characterised as super infective as it out-competes other strains of *P. aeruginosa* in the CF lung. Interestingly, this bacterium has been reported to colonise non-CF patients/CF carers and it has been shown to cross the species barrier as it has also been isolated in a CF patients cat (Mohan *et al.*, 2008). Ashish (2012) showed the increase in resistance of LES to standard anti-pseudomonal antibiotics from 2004 to 2008; figure 1.3.

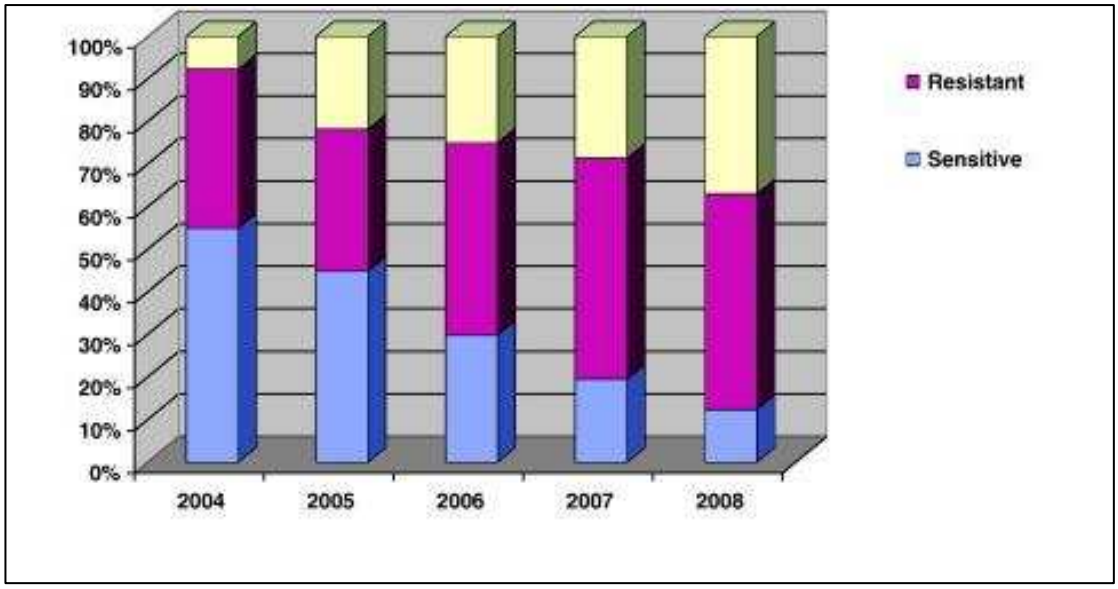


Figure 1.3: Increase in resistance of LES to standard anti-pseudomonal antibiotics.

The period from 2004 to 2008 is shown and it shows the increase in resistant isolates and the decrease in sensitive isolates to current antibiotics (Ashish *et al.*, 2012).

1.5.4. Bacterial cross infection in the chronic lung environment

The first case of bacterial cross infection observed between chronic respiratory disease patients was in 1984 when 11 patients in a CF clinic based in Leeds, UK were infected with the same strain of *Pseudomonas cepacia* (*P. cepacia*) (Simmonds *et al.*, 1990). However, it was not generally accepted that the isolation of this bacterium in all 11 patients was due to cross infection until 1993. It was more envisaged that each patient had become colonised with *P. cepacia* individually (Govan *et al.*, 1993, Smith *et al.*, 1993). In the early 1970's, CF patients were treated at CF summer camps and it was observed that the frequency of *P. cepacia* being isolated from patients steadily increased as time progressed; 10 % of patients in 1971 compared to 18 % of patients in 1981 (Thomassen *et al.*, 1986, Isles *et al.*, 1984). In 1984, it was shown that this increase in patients becoming colonised with *P. cepacia* was leading to higher morbidity rates (Isles *et al.*, 1984). Even though the premise of bacterial cross infection between CF patients pre-1993 was not proven, some hospitals from 1983 onwards did treat CF patients colonised with *P. cepacia* separately to culture negative individuals. These interventions and precautions are still followed in all UK hospitals. In 1992, *P. cepacia* was reclassified as *Burkholderia cepacia* (*B. cepacia*). In 1997, it was found through serotyping that *B. cepacia* was in fact split into at least 17 genetically distinct species groups, which is why this bacteria is often referred to as *B. cepacia* complex C (Coenye *et al.*, 2001, Vermis *et al.*, 2002, Eram *et al.*, 2004, Rushton *et al.*, 2013, LiPuma, 2010).

Cross infections occurring in the BR lung are not as well-known but it is premised that *P. aeruginosa* will behave in a similar manner. BR patients are not always treated in hospitals according to their bacterial colonisation as they are often being treated in various wards relating to other health problems, which normally are a result of increased patient age (personal communication).

1.6. BACTERIOPHAGE/PHAGE

Francis Twort and Felix d'Herelle are given the honour of independently discovering bacteriophages in the 1900's (d'Herelle, 1917, Twort, 1915). They both observed altered growth in the bacterial strains they were investigating and named these entities 'bacteria eaters' or 'bacteriophages'. These 'bacteria eaters' were described as entities that could be filtered away from bacteria yet still eradicate subsequent bacterial populations (d'Herelle, 1917, Twort, 1915). Sulakvelidze *et al* (2001) detailed that there were in fact two earlier discoveries of bacteriophages but neither labs followed up their discoveries. A British bacteriologist Hankin in 1896 reported the presence of marked antibacterial activity against *Vibrio cholera* by an unidentified substance (Hankin, 1896). Two years later a Russian bacteriologist Gamaleya, also observed a similar phenomenon but in this instance the bacterial background was *Bacillus subtilis* (Samsygina and Boni, 1984, Sulakvelidze *et al.*, 2001, Van Helvoort, 1992). As neither labs followed up this research, it allowed the honour of discovering bacteriophages to be accredited to both Twort and d'Herelle (Sulakvelidze *et al.*, 2001).

Bacteriophages are thought to be the most abundant biologically related entity in nature, outnumbering bacterial cells in a ten to one ratio (Fineran *et al.*, 2009, Chibani-Chennoufi *et al.*, 2004, Wommack and Colwell, 2000, Ashelford *et al.*, 2003). Recent estimates using epifluorescence microscopy has indicated that the amount of viruses in the ocean spans from ~ 10^6 mL^{-1} in the deep sea to ~ 10^8 mL^{-1} in coastal waters (Suttle, 2005, Guixa-Boixereu *et al.*, 2002, Ortmann and Suttle, 2005).

In the last century, phage research has shown that these viral entities are extremely diverse and ubiquitous in the biosphere. In 1987, it was described that there were around 4,500 different double stranded DNA phages which were capable of infecting a wide range of Gram negative and Gram positive bacterial hosts (Coetzee, 1987, Hendrix *et al.*, 1999). Studies have also shown that any environment harbouring bacterial cells also allows for the isolation of bacteriophages (Hendrix *et al.*, 1999, Ackermann, 1996, Bergh *et al.*, 1989).

Phages can also be isolated from a range of environments spanning from marine ecosystems to the human gut, showing the wide spanning nature of bacteriophages in environmental and clinical settings (Paul *et al.*, 2002, Breitbart *et al.*, 2008). Free phage has been isolated in sputum of CF patients at densities which were directly proportional to the levels of LES within the sputum (James *et al.*, 2015). LES contains multiple prophages (Winstanley *et al.*, 2009) however, James *et al* (2015) identified that phage 2 and 4 were most abundant in the sputum of the 10 LES infected patients studied. This is not the first finding of free phage in the sputum of CF patients (Ojeniyi *et al.*, 1991, Fothergill *et al.*, 2011) so the study presented by James *et al* (2015) adds strength to the hypothesis that free phage are widely identifiable in clinical settings.

1.6.1. Bacteriophages and their life cycle

1.6.1.1. Lytic bacteriophages

Lytic bacteriophages cannot integrate into the bacterial chromosome and thus, are truly vegetative in nature. They infect their sensitive bacterial host, replicate and then lyse this host cell. This lysis allows their transmission into the surrounding cellular environment where the progeny viruses can infect other sensitive host cells and the circle can be repeated. Table 1.4 details some of the lytic bacteriophages of *P. aeruginosa* however, this is not an exhaustive list.

1.6.1.2. Temperate or lysogenic phages

Bacteriophages have evolved 2 different life cycles in order to alter how they infect and proliferate within their bacterial host cells. These life cycles are termed lytic and lysogenic and are detailed in figure 1.4. Temperate/lysogenic bacteriophages upon infection of a sensitive host cell have the ability to integrate their viral genome into the chromosome of the recipient bacteria. This integrated phage, now termed a prophage, is replicated alongside any other bacterial chromosomal loci. These prophage regions remain site specifically integrated in the bacterial chromosome until an environmental stimulus induces the cellular SOS system and Rec mediated response (typical method in lysogenic bacteriophage). However, certain phages have been shown to integrate into multiple sites within the bacterial chromosome (Fogg *et al.*, 2010) whereas, other phages can only integrate into their bacterial chromosome at specific sites. Table 1.5 details some of the temperate bacteriophages of *P. aeruginosa.*

Bacteria have been shown to have genomes studded with multiple prophages. The Gram negative bacteria *P. aeruginosa* and *E. coli* have been shown to harbour multiple inducible prophage regions (Winstanley *et al.*, 2009, George *et al.*, 1975, Bertani, 1951, Perna *et al.*, 2001). The Gram positive bacteria *S. aureus* also has been seen to contain prophage regions (Goerke *et al.*, 2009, Gill *et al.*, 2005). Ramirez and colleagues found that around 75 % of the strains of *Streptococcus pneumoniae* harboured prophage regions (Ramirez *et al.*, 1999, Obregón *et al.*, 2003).

Table 1.4: Examples of some lytic bacteriophages which can target *P. aeruginosa.*

This is not an exhaustive list of all the bacteriophages present in the environment with *P. aeruginosa* as a host.

Lytic bacteriophage	Reference
LKD16	(Ceyssens *et al.*, 2006)
LKA1	(Ceyssens *et al.*, 2006)
PA1Ø	(Kim *et al.*, 2012)
MPK1	(Heo *et al.*, 2009)
MPK6	(Heo *et al.*, 2009)
JG004	(Garbe *et al.*, 2011)
BVPaP-3	(Ahiwale *et al.*, 2012)

Table 1.5: Examples of some temperate bacteriophages which can target *P. aeruginosa.* This is not an exhaustive list of all the bacteriophages present in the environment with *P. aeruginosa* as a host.

Temperate bacteriophage	Reference
Pan70	(Holguín *et al.*, 2015)
PaMx73	(Cazares *et al.*, 2014)
H70	(Cazares *et al.*, 2014)
D3112	(Roncero *et al.*, 1990)
B3	(Roncero *et al.*, 1990)
F116	(Holloway *et al.*, 1960)
ϕCTX	(Byrne and Kropinski, 2005)

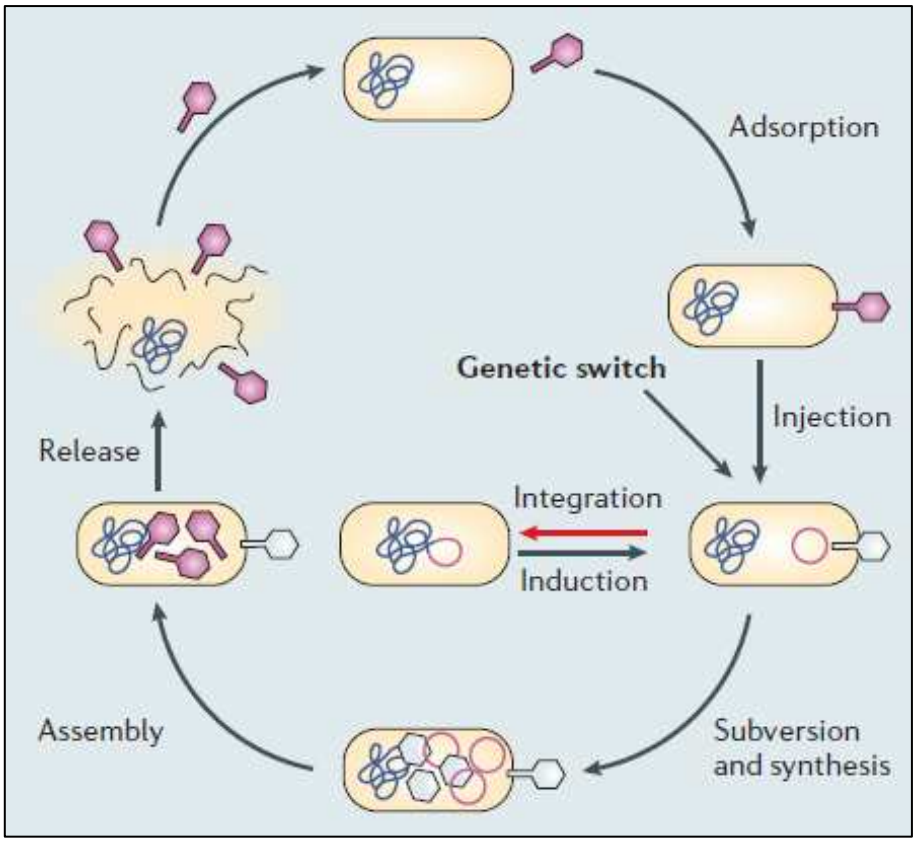


Figure 1.4: Lytic and lysogenic pathway of phage infection (Sturino and Klaenhammer, 2006). This image shows the two pathways that phages can follow upon infection of a sensitive host. The lysogenic pathway shows the incorporation of the phage genome into the bacterial chromosome forming prophage regions. These prophages remain integrated into bacterial chromosome until the bacterium is stressed (UV/antibiotics), these regions are then induced from the bacterial chromosome. The phage particles upon induction from the bacterial chromosome enter into the lytic lifecycle causing host cell lysis and the release of infective phage particles which can spread and infect all the susceptible hosts within a particular niche (Sturino and Klaenhammer, 2006).

1.6.2. Lambdoid-like phages

The typical phage lambda (λ) model of *E. coli* is described in detail here and this genetic model was initially proposed in the 1950's (Fry, 1959, Lieb, 1953). The relationship between lysogenic conversion and the induction of the lytic cycle is very complex and involves multiple phage encoded proteins. Recently, new phages have been discovered which challenge the conventional mechanism. For example, some phages have the ability to re-infect their originating host cell so indicating that prophage carriage doesn't prevent further phage infection in some bacterial species (Smith *et al.*, 2012, Tariq *et al.*, 2015). This phenomenon is further described in chapter 3.

1.6.2.1. Phage adsorption and infection

Ever since the initial observation of phage particles by electron microscopy in the 1940's, it has been possible to study the mode of adsorption that phages utilise in order to infect their susceptible host (Ruska, 1941, Luria and Anderson, 1942, Luria *et al.*, 1943, Bayer, 1968). In the typical *E.coli* λ model, the phage DNA is injected into the host's chromosome via the phages tail protein, J. J interacts with LamB, which permits the entry of phage DNA into the bacterial host cell (Ptashne, 2004, Turner *et al.*, 2007, Ptashne and Switch, 1992, Rakhuba *et al.*, 2010, Randall-Hazelbauer and Schwartz, 1973). Pilus dependant filamentous and RNA phages have been found for *P. aeruginosa* (Feary *et al.*, 1964, Bradley, 1966, Takeya and Amako, 1966, Olsen and Shipley, 1973, Bradley and Pitt, 1974). Phage DNA is normally injected into the bacterial host cell as a linear segment which can re-circularise upon entry through the use of *cos* sites and host DNA ligase.

1.6.2.2. Phage life cycle decision – genetic regulation of lambdoid phage infection

Host DNA gyrase causes the supercoiled DNA to unwind which allows for transcription to commence. Immediate early transcripts utilise phage encoded promoters; P_L, P_R and P_R'. These transcripts lead to the expression of *N* and *Cro.* Cro functions by binding to the O_R3 site on P_{RM} which stops any P_{RM} expression of CI. N functions via two N utilisation (*Nut*) sites; one binds to the P_L reading frame and the other one binds to the *cro* gene region in P_R. N is an anti-terminator protein that allows for the expression of the reading frame to which it is bound. It does this by recruiting the host's RNA polymerase which causes the expression of 'late early' gene transcripts; *N, cro, CII, CIII, xis, int, O, P* and *Q* (McLennan *et al*., 2012, Ptashne, 2004). CII and CIII are substrates for FtsH, a bacterial protease. CIII can prevent CII from being degraded since it acts as an alternative substrate (Shotland *et al*., 1997, Shotland *et al*., 2000). CII stability determines whether the phage enters into either a lytic or lysogenic lifecycle. When CII is available there is a propensity for lysogeny and lysis occurs if CIII and CII levels are depleted (Michalowski and Little, 2005). Figure 1.5 describes and details some of the genes involved in the phage life cycle decision process (Smith *et al*., 2007). The integration site of the prophage region can provide the bacterial host with selective advantages due to lysogenic conversion. Lysogenic conversion is described in more detail in chapter 3, but the principle is that the diversity of the bacterial host is elevated upon prophage formation and this may provide the host with a selective advantage compared to non-lysogenic/naïve bacterial cells. The expression of moron/accessory genes from the phage chromosome may also offer a selective advantage to both the bacterium and the phage.

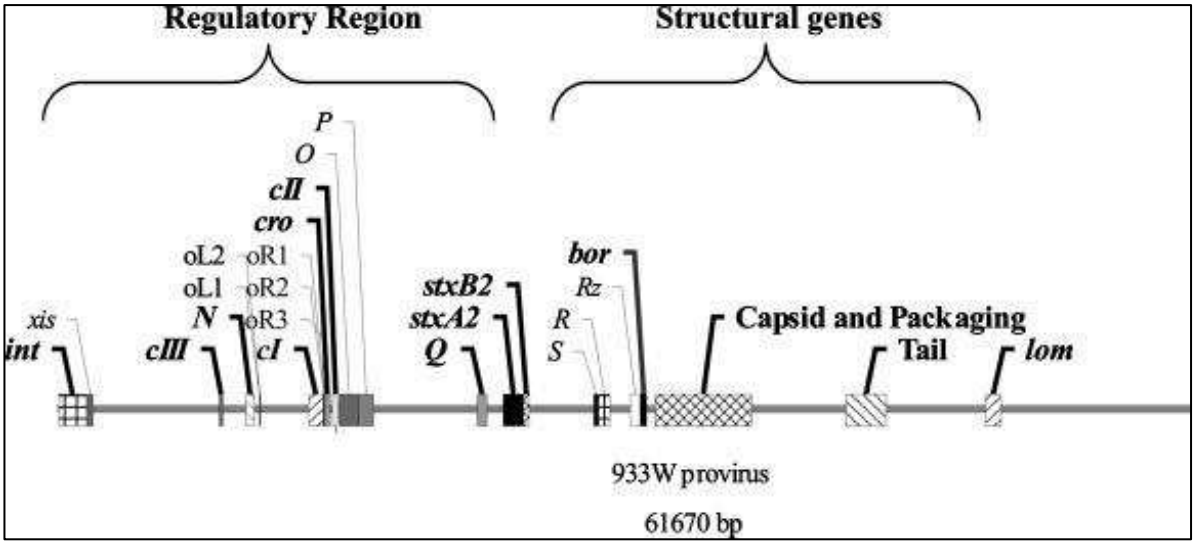


Figure 1.5: Linear genome organisation of the well-studied stx phage, 933w (Smith *et al.*, 2007). Upon entry of the phage genome into the *E. coli* host cell, the phage protein J interacts with LamB which allows for internalisation of the phage genome. The phage genome upon internalisation is in a linear format with *att* sites located at either end of the genome (not shown here). The annotations shown on this figure are: *Int* – integrase gene, *Xis* – excisionase gene, *CIII* – involved in the stabilisation of CII, *N* –early gene regulation role (its two operator sites are shown as oL1 and oL2), *CI* - *λ* repressor which inhibits iniation of the lytic lifecycle, *cro* – repressor involved in the regulation of the lytic lifecycle, *CII* – regulator of *λ* integrase synthesis and the *λ* repressor, *O* and *P* – phage DNA replication machinery, *Q* – late gene regulation, *stxA2* and *stxB2* – toxin genes. All the genes that fall under the bracket of structural genes are involved in the formation of the capsid, head, tail spike and the tail fibre.

1.6.2.3. Lysogenic lifecycle

When CII reaches saturation levels it becomes stable, which in turn leads to the activation of its own promoters and this activation generates lysogeny and stops 'late early' expression (Herskowitz and Hagen, 1980). CII promotes transcription from P_{RE}, P_I and P_{antiq}; P_{RE} causes the O_R3 site on P_{RM} to be released by Cro which can therefore, also promote the transcription of CI. P_I encodes for integrase (int) which contributes towards the incorporation of a lysogenic phage into the bacterial chromosomal backbone to form a prophage region. P_{antiq} switches off *Q* (anti-terminator) and stops late gene transcription thus, preventing expression and assembly of lytic and structural proteins. CI produced by P_{RE} expression stabilises both the P_R and P_L promoters so therefore, ensuring that Cro is continually turned off. The operator regions of these promoters bind to both the O_R1 and O_R2 sites on Cro, which allows for constant CI expression. Once CI expression is established the promoters, P_R and P_L are switched off. This leads to a decrease in the production of CII/CIII and an overall reduction in transcription from the 'late early' gene promoters. The only promoters that remain active during lysogeny are involved in the maintenance of CI; P_{RM} and $P_{R'}$ (McLennan *et al.*, 2012, Ptashne, 2004).

1.6.2.4. Maintenance of the lysogenic state

When CI binds to the O_R1 site on Cro it prevents any further transcription from P_L thus, causing any transcription to be terminated. CI can easily dimerise so upon binding to the O_R1 site it can also bind to the O_R2 site. This dimerisation leads to transcription commencing from P_{RM} in the opposite direction. This expression can activate $P_{R'}$ which ensures that only CI is transcribed when the phage is residing in the bacterial chromosome as a prophage (McLennan *et al.*, 2012, Ptashne, 2004).

1.6.2.5. Induction of the prophage

When the host bacterium is stressed by external stimuli such as antimicrobials/antibiotics/UV radiation, the SOS response is activated and in most instances this leads to the activation of RecA. RecA* in lysogenic cells cleaves the CI dimer, CI upon cleavage loses its affinity for DNA. This means that the P_R and P_L promoters are no longer repressed and the phage can enter into the lytic lifecycle. Upon induction from the prophage region, temperate phages switch to lytic infection and cause host cell lysis in a manner similar to purely lytic bacteriophages. In order for the phage to be excised from the bacterial host chromosome a phage encoded integration protein, Xis, is required (Fogg *et al.*, 2014).

1.6.2.6. Lytic lifecycle

Purely lytic bacteriophages and temperate phages that have been induced from their bacterial host cell chromosome follow the same pathway as soon as they enter into lysis. O binds to the origin of replication (ori) which determines the direction of phage expression. Then another product of late early gene expression, P, binds to DnaB (part of the *E. coli* replication machinery) and establishes lytic phage infection. Q (late early gene expression product) is also involved in lytic replication, as this protein binds to Q utilisation (Qut) sites on RNA polymerase which promotes lytic phage replication. P_R' expression continues throughout lytic phage infection, which generates mRNA for the production of the lysis, head and tail proteins. These structural proteins can then assemble to form new infective phage particles which can be excised from the *E. coli* genome via the production of holin. Holin aids the release of the phage

particles whilst also promoting host cell lysis (McLennan *et al.*, 2012, Ptashne, 2004).

1.6.2.7. Mosaic layout of lambdoid-like phages

Genetic mosaicism was identified for the first time in the later 1960's/early 1970's within the lambdoid phages isolated from enteric environments (Simon *et al.*, 1971, Westmoreland *et al.*, 1969, Pedulla *et al.*, 2003b). It was clear from these genetic studies that there were mosaic joints within the phage genomes and the occurrence of these joints was not due to random integration events. It was proposed that these joints occurred between genes or conserved clusters of genes on the phages backbone (Pedulla *et al.*, 2003b).

Mosaic genomes have also been seen in mycobacteriophages; L5, D29, Bxb1 and TM4 (Pedulla *et al.*, 2003b, Ford *et al.*, 1998a, Ford *et al.*, 1998b, Hatfult and Sarkis, 1993, Mediavilla *et al.*, 2000). Even though these phages were isolated at different geographic locations and at different times, the phages have many features in common including morphology, genome size, structure and assembly gene organisation and related gene products (Pedulla *et al.*, 2003b). L5 and D29 have been shown to share over 75 % of their genes based on amino acid similarity, so showing the mosaic and modular nature of bacteriophages (Ford *et al.*, 1998a, Pedulla *et al.*, 2003b).

1.6.3. Bacteriophage of *Pseudomonas*

The International Committee on the Taxonomy of Viruses (ICTV) defines that the temperate phage of *Pseudomonas* can be subdivided into tailed and non-tailed morphologies and can also be categorised according to their core genetic

backbone. The vast majority of *Pseudomonas* phage (over 97 % in 2010) had a morphology that represented the order *Caudovirales*. These phages have a dsDNA genome and are tailed. Around 3 % of the described *Pseudomonas* phage have a polyhedral, filamentous or pleomorphic morphology (PFP) (Ceyssens and Lavigne, 2010). The *Caudovirales* can be subdivided into three families (Myoviridae, Podoviridae and Siphoviridae). Some of the most common morphologies of tailed *Pseudomonas* phage are shown in figure 1.6.

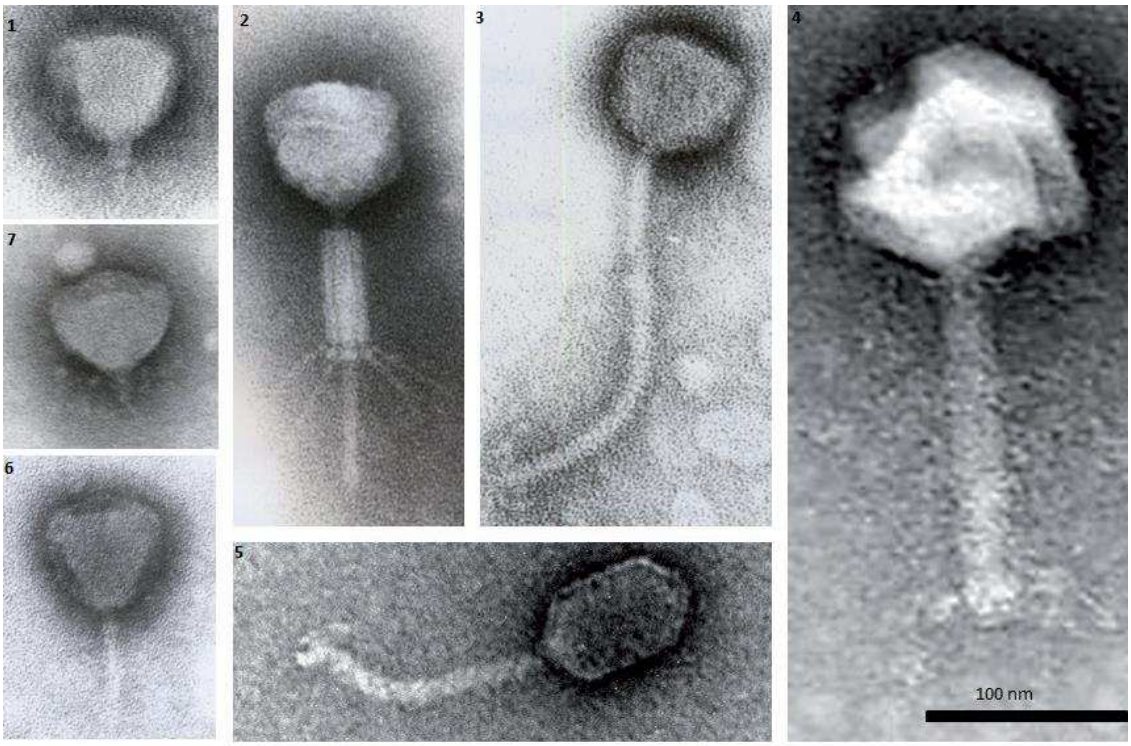


Figure 1.6: Phenotypic differences observed in the tailed phages of *Pseudomonas* (Ceyssens and Lavigne, 2010). This is a representation of some of the most common tailed phages of *Pseudomonas* which had been isolated and characterised by 2010. A scale bar is included in order to scale the phage particles. 1) LUZ24 like phage LUZ14, 2) PB1-like phage LBL3, 3) D3112–like phage LAD2, 4) ϕ KZ, 5) YuA, 6) F116–like phage LL02 and 7) ϕKMV–like phage LUZ2.

1.6.3.1. *Caudovirales* phages of *P. aeruginosa*

1.6.3.1.1. Myoviridae phages of *P. aeruginosa*

The Myoviridae family all have long, helical tails that contract upon infection of their bacterial host. Within the Myoviridae, three distinct types of phage groups have been isolated which all have highly discrete genome architectures; these are phage KZ, phage CTZ and phage PB1 (Ceyssens and Lavigne, 2010). Phage KZ, a jumbo phage, was originally isolated in 1975 in Kazakhstan (Krylov *et al.*, 1977). It was one of first of the large genome phages that can infect *P. aeruginosa* to be characterised. Jumbo phages have more than 200 bp of DNA and the majority of the predicted proteins from these phages have no similarity to known phage proteins (Hendrix, 2009). Phage KZ has a circular genome of 280 kb, which means that it fits into the range of jumbo phage genomes that are seen in other bacterial species (210 – 316 kb) and it also encodes for between 201 – 461 genes (Ceyssens and Lavigne, 2010). Phage CTZ is a temperate, cytotoxin converting myovirus that infects the *P. aeruginosa* strain, PAS10, via the 3' end of the bacterial serine transfer RNA (tRNA) (Hayashi *et al.*, 1990). It has a 35 kb genome and produces CTX, a pore-forming cytotoxin that increases host virulence (Baltch *et al.*, 1994). Phage PB1 represents another distinct group of the Myoviridae phages of *P. aeruginosa* and it is composed of at least 22 different proteins.

1.6.3.1.2. Siphoviridae phages of *P. aeruginosa*

Members of the Siphoviridae family are characterised by long and non-contractile tails. All the family members identified to date (2010) are temperate phages of *P. aeruginosa* (Ceyssens and Lavigne, 2010). With the most characterised examples being D3112; this is a type IV pili specific phage that can replicate its

40 kb genome by transposition. Interestingly, on excision from their bacterial host's genome they accrue part of the bacterial host chromosome as it is incorporated inside the newly formed viral particles (Morgan *et al.*, 2002). This misexcision aids the horizontal dissemination of genetic material throughout the phages host range. Phage D3, even though it is morphologically identical to D3112 its genome is larger (56 kb). D3 is also not transposable and it contains two distinct integration sites for prophage formation (Ceyssens and Lavigne, 2010). YuA–like phage are also *Siphoviruses* but they are not as well studied, they do however, have elongated head sections which are similar to the ones identified when studying phage D3112 (Ceyssens and Lavigne, 2010).

1.6.3.1.3. Podoviridae phages of *P. aeruginosa*

Pseudomonas-specific *podoviruses* have short tails and only display minor differences in terms of capsid size and tail structure. Phage KMV is a *podovirus* which has a 42 kb genome. Recently, other phages have been isolated with similar sequence homology and these phages only carry minor insertions and deletions upon comparison to the original phage KMV genome. Examples of these phages are LKD16, LUZ19, PT2, PT5 and KF77; these phages share between 83 and 97 % homology with the KMV phage (Kulakov *et al.*, 2009, Lammens *et al.*, 2009, Ceyssens *et al.*, 2006, Ceyssens and Lavigne, 2010). F116 and LUZ24-like phages are also associated with the *Podovirus* family; F116 is unusual as it replicates as a pseudo-lysogen within its host cell, as it forms a plasmid rather than integrating into the host chromosome to form a prophage (Byrne and Kropinski, 2005).

1.6.3.2. Polyhedral, filamentous or pleomorphic morphology phages of *P. aeruginosa*

The Inoviridae family are rod shaped, single stranded DNA (ssDNA) filamentous phages that adhere to their bacterial hosts via the pilus (Ceyssens and Lavigne, 2010). The longest filamentous phages that have been found to infect *P. aeruginosa* are Pf1 and Pf3. Pf prophage sequences are common in *P. aeruginosa* strains and can account for some of the variation present between the multiple *Pseudomonas* strains (Ceyssens and Lavigne, 2010). Pf4 for example, has been shown to be involved with the appearance of SCV's in PAO1 mature biofilms (Webb *et al.*, 2004). The Cystoviridae are a family of the filamentous phages which have a double stranded RNA (dsRNA) genome encased within a lipid-containing membrane (Mindich *et al.*, 1999). The Leviviridae family is the final family within these filamentous phages; these have positive sense single stranded RNA (ssRNA) genomes. These genomes are encased within an icosahedral capsid and they also have relatively small genomes of approximately 3.5 x 10^2 nucleotides (Persson *et al.*, 2008, Ceyssens and Lavigne, 2010).

1.6.4. Prophage integration into susceptible bacterial hosts

The incorporation of a prophage region increases the genetic size of the bacterial host and can offer positive or negative pressures which may aid selection for the bacterium. Desiere *et al* (2001) proposed that phage infection must offer a selective advantage for the bacterium otherwise Darwinian considerations would lead to the assumption that prophage inclusion would decrease bacterial fitness levels. It is proposed that prophage inclusion can increases the metabolic burden of the bacterial host due to the addition of extra DNA into the host bacterium. This therefore, makes replication more costly which reinforces the hypothesis that prophage regions must be advantageous for the host in order to compensate for this increased cost (Desiere *et al.*, 2001, Ferretti *et al.*, 2001). This increase in genome size may play a role in the evolutionary pathway of the bacteria. These potential evolutionary changes may be due to gene alterations which are generated as a result of transduction between the phage and the bacterium (Weinbauer and Rassoulzadegan, 2004).

This genome size alteration is seen in both *P. aeruginosa* and *E. coli* upon phage incorporation. Winstanley *et al* (2009) showed that the LES genome (6.6. Mb) contained 5 inducible prophage regions and 1 defective prophage region. This is in contrast to the lab strain, PAO1, which has a smaller genome size of 6.3 Mb and is believed to harbour only two prophage regions which may explain the reduced genome size (Stover *et al.*, 2000). The pathogenic strain of *E. coli* O157:H7, which originates from an *E. coli* outbreak within school children in Sakai, Japan has been shown to contain 18 prophages and prophage-like elements however, not all these phages are inducible (Ohnishi *et al.*, 2001). This shigatoxigenic *E. coli* isolate has a genome size of 5.5 Mb (Hayashi *et al.*, 2001) which is considerably larger than the lab strain of *E. coli* K12 which has a genome size of 4.6 Mb (Blattner *et al.*, 1997, Lawrence and Ochman, 1998).

1.6.5. Bacterial and phage resistance mechanisms

As a key focus of the research in this thesis is on the evolving interactions between the phage and its bacterial host thus, the ways in which bacteria prevent phage infections are important to understand. There are multiple anti–phage mechanisms described to date that limit bacterial sensitivity to viruses and will be described in detail in the next few sections. Detailed descriptions of the various mechanisms bacteria have evolved in order to reduce phage infection are shown in sections 1.6.5.1 to 1.6.5.5.

1.6.5.1. Preventing phage adsorption

In order to prevent phage adsorption, bacteria can alter their surface structures to prevent phage internalisation as phages wouldn't recognise these new adsorption domains. In 1983, is was shown that phage resistant strains of *Streptococcus lactis* were resistant to phage infection by M18 (*Streptococcus cremoris* phage) due to alterations in the cell surface receptor sites, these sites therefore, altered phage adsorption (Sanders and Klaenhammer, 1983, King *et al.*, 1983). Bacteria have also evolved mechanisms that prevent phage DNA injection into the bacterial chromosome thus preventing, lysis or prophage formation.

1.6.5.2. Restriction modification (RM) systems

RM systems were proposed by Arber and described by Smith and Welcox (Kühnlein and Arber, 1972, Smith and Welcox, 1970, Blumenthal and Cheng, 2002). RM systems are one of the best studied host defence systems (Tock and Dryden, 2005, Stern and Sorek, 2011). They are composed of 2 components which restrict and modify the DNA entering into a bacterial host cell. Their main

function is to provide the bacterial host cell with protection cell against incoming foreign DNA, such as bacteriophage DNA (Arber, 1979, Pingoud and Jeltsch, 2001). The restriction aspect of the system involves an endonuclease breaking the DNA backbone of the invading DNA sequence and generating a dsDNA break (Blumenthal and Cheng, 2002). The modification aspect involves a methyltransferase which ensures that non-foreign DNA is methylated and so is protected against restriction activity (Blumenthal and Cheng, 2002). Figure 1.7 details the functionality of a typical type II RM system (Stern and Sorek, 2011). There are three types of RM system currently known which are characterised and identified according to their cofactor requirements, mode of action and subunit composition (Smith and Nathans, 1973, Pingoud and Jeltsch, 2001).

Over 3,000 RM systems had been discovered by 2002 and they had been found in the full spectrum of known bacterial systems (Blumenthal and Cheng, 2002). Over 90 % of sequenced bacterial and archaea genomes contain RM systems (Roberts *et al.*, 2010, Stern and Sorek, 2011). *Haemophilus influenzae, Methanococcus jannaschii, Helicobacter pylori, Neisseria gonorrhoeae* and *Neisseria meningitids* contain multiple RM systems whilst *Treponema pallidum, Chlamydia* and *Buchnera* almost always lack RM genes (Stein *et al.*, 1995, Tomb *et al.*, 1997, Alm *et al.*, 1999, Kobayashi, 2001).

Phage can counteract the effects of these systems through various alterations such as mimicking the DNA of the host and thus, not being detected by the host's RM system. The Ocr protein of phage T7 blocks the active site of some of the restriction enzymes in *E. coli* RM systems through mimicking a 24 bp fragment of DNA and this prevents the degradation of the invading phage DNA (Bandyopadhyay *et al.*, 1985, Stern and Sorek, 2011). Phage 1 of *Bacillus subtilis* would be recognised by its host RM system due to the non-methylated sequence CGCG. However, the phage can alter some of the bases within this

detection site, thus preventing its detection (Krüger and Bickle, 1983, Stern and Sorek, 2011, Blumenthal and Cheng, 2002). Figure 1.7 describes the functionality of RM systems whilst figure 1.7B details some mechanisms that can be utilised by phages in order to avoid host RM systems (Stern and Sorek, 2011).

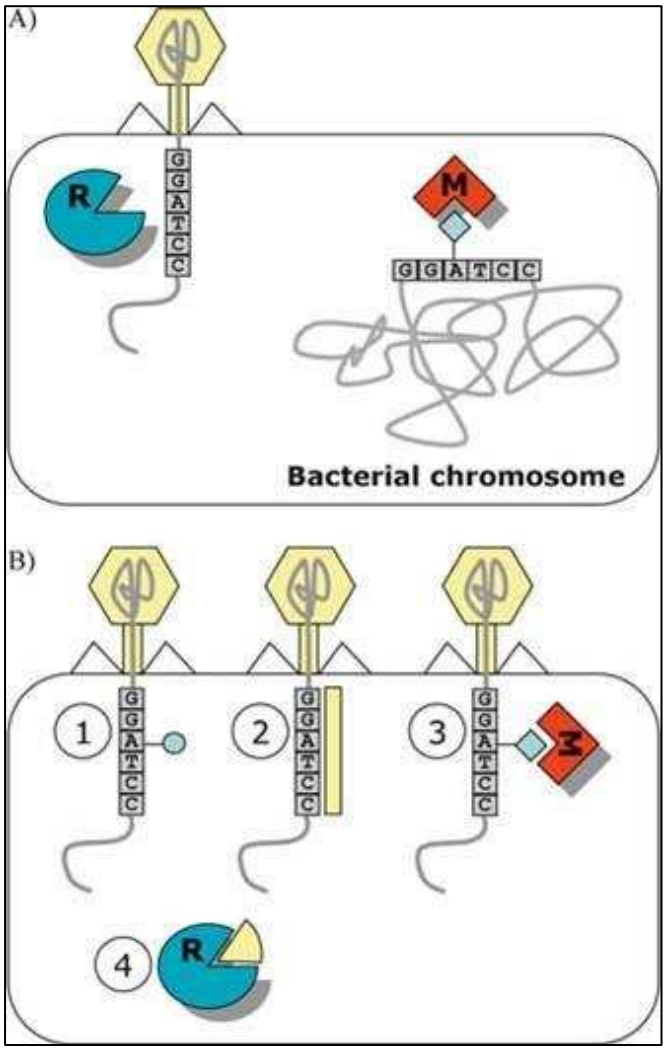


Figure 1.7: Type II RM systems: panel A shows the functionality of a typical type II system whilst panel B shows the variety of ways that phage can evolve in order to avoid the RM systems (Stern and Sorek, 2011). The type II RM system is shown in panel A (R – restriction enzyme and M – methytransferase). It shows that non-methylated DNA sequences are recognised as foreign, whilst methylated DNA is incorporated into the bacterial host chromosome as it is not detected by the RM system. Panel B shows the various ways in which phages have evolved in order to prevent their detection by host RM systems. 1) Incorporation of an unusual base which prevents the detection of the foreign DNA (Krüger and Bickle, 1983). 2) Masking the restriction enzyme site on the phage DNA by a phage protein (Iida *et al.*, 1987). 3) Stimulation of methyltransferase activity by the incoming phage. 4) Neutralisation of the restriction enzyme by a phage protein mimicking the DNA to which the enzyme normally binds (Dryden and Tock, 2006, Stern and Sorek, 2011).

1.6.5.3. CRISPR/Cas systems

CRISPR/Cas systems are small, RNA based defence systems that can provide either a bacterial/archaeal system with adaptive and heritable immunity against invading foreign DNA/RNA (Terns and Terns, 2011). CRISPR/Cas systems were initially described in 1987, when a Japanese group led by Ishino discovered a set of short tandem repeats in *E. coli* (Ishino *et al.*, 1987, Deveau *et al.*, 2010). CRISPR are composed of repeat sequences that can range from between 21 to 48 bp. These repeats are separated from one another by spacers that can span from 26 to 72 bp (Barrangou *et al.*, 2007, Bhaya *et al.*, 2011, Cady *et al.*, 2012). The sequences of these spacer regions are variable but are often 100 % identical to the genetic sequence of frequently encountered bacteriophages, plasmids or other foreign nucleic material (Van Der Oost *et al.*, 2009, Cady *et al.*, 2012).

Cady *et al* (2012) investigated the genomes of 122 clinical isolates of *P. aeruginosa* and determined that 36 % of these isolates harboured CRISPR/Cas systems. It was also found that many of these CRISPR/Cas systems were 100 % identical to *P. aeruginosa* chromosomal integrating mobile genetic elements. This similarity supports the idea that CRISPR/Cas systems function to detect and cleave foreign nucleic acid thus, protecting the bacteria or archaea from deleterious effects due to the incorporation of a phage or plasmid.

Three stages have been proposed in the CRISPR/Cas pathway; adaptation of the CRISPR system to the invading nucleic acid, biogenesis of mature transcripts and finally, silencing of the invader sequence (Terns and Terns, 2011). Copies of the foreign nucleic material (protospacer sequence) are initially acquired from an invading entity such as a bacteriophage/plasmid and this protospacer sequence becomes integrated into the host CRISPR locus. This integration is controlled by the protospacer adjacent motifs (PAM) located on the invading DNA sequence (Terns and Terns, 2011). The CRISPR regions are then transcribed as one long

transcript prior to processing into shorter and mature CRISPR RNAs (crRNAs). This processing either is undertaken by CRISPR associated (Cas) proteins or by Cas proteins combined with host RNase III (Brouns *et al.*, 2008, Deltcheva *et al.*, 2011, Haurwitz *et al.*, 2010, Cady *et al.*, 2012). The mature crRNAs transcripts are then complexed with subtype specific Cas proteins. These complexes can then interact with the foreign nucleotide sequences, these sequences are complementary to the CRISPR spacer sequences and so generate innate immunity towards the invading DNA sequence (Brouns *et al.*, 2008, Hale *et al.*, 2009, Jinek *et al.*, 2012, Wiedenheft *et al.*, 2011, Cady *et al.*, 2012). A schematic of the functionality of a type II CRISPR/Cas system is shown in figure 1.8 (Samson *et al.*, 2013).

Phages, to counteract the effects imposed upon them by CRISPR/Cas sytems, can mutate the region of their genome which the bacterial CRISPR/Cas system detects or even in extreme cases delete this section (Deveau *et al.*, 2008). Alternatively, the phage can develop anti-CRISPR genes (Bondy-Denomy *et al.*, 2013), so ensuring that detection by the bacteria cannot occur.

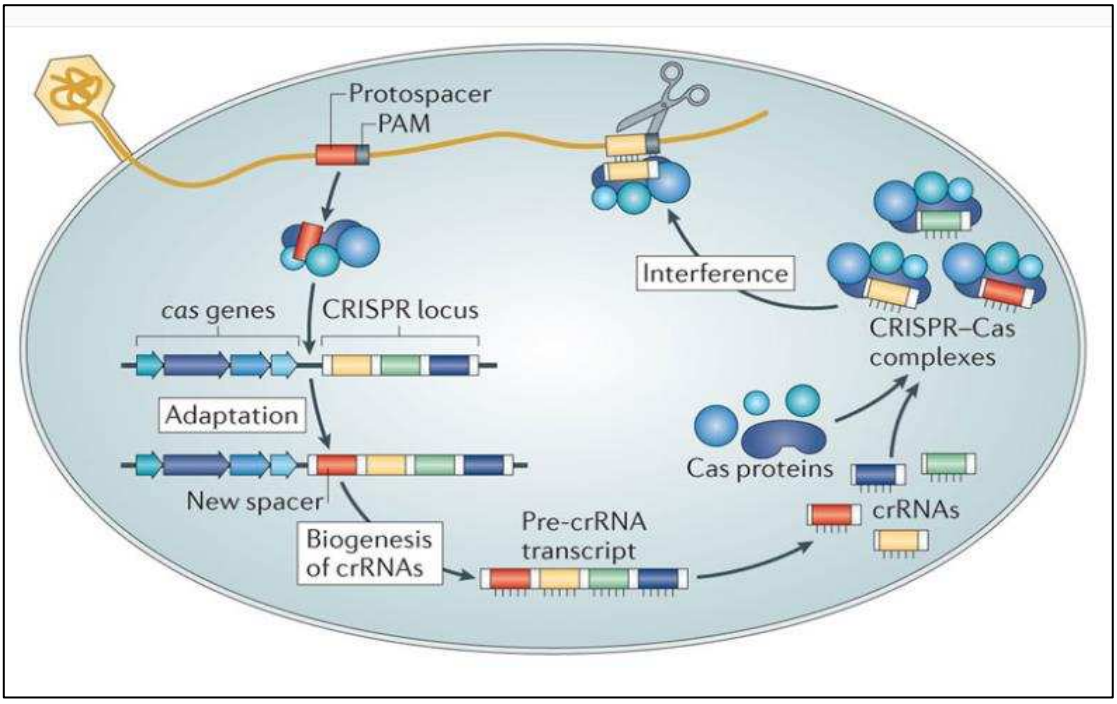


Figure 1.8: CRISPR/Cas functionality (Samson *et al.*, 2013). A type II CRISPR/Cas system is shown in this figure and it shows the three general steps that are involved in all CRISPR/Cas defence systems; (1) adaptation involving the acquisition of spacer sequences, (2) biogenesis of crRNA and CRISPR-Cas complexes and finally, (3) interference which involves the cleavage of the invading phage DNA. All these process prevent the formation of either a prophage due to lysogenic phage activity or host cell lysis due to lytic phage activity (Samson *et al.*, 2013). Type I CRISPR/Cas systems involve Cas3 in the expression and biogenesis step (Makarova *et al.*, 2011, Sinkunas *et al.*, 2011). Type II CRISPR/Cas systems involve RNAse II and tracrRNA (*trans* encoded small RNA) whilst type III systems utilise Csm or Cmr proteins (Makarova *et al.*, 2011).

1.6.5.4. Toxin Antitoxin (TA) systems

TA defence systems are either based upon programmed cell death or the induction of dormancy within the prokaryote in response to infection by foreign nucleic material (Makarova *et al.*, 2013). TA systems are widespread in bacteria and archaea. These systems have evolved in order to enhance bacterial and archaeal survival through promoting persistence, maintaining specific genetic loci whilst also providing resistance to phage infection (Blower *et al.*, 2012).

TA systems typically consist of two genes encoded within a single operon, these genes encode for both a stable toxin (disrupts an essential cell process) and an unstable antitoxin (controls toxicity levels within a prokaryotic host) (Wang and Wood, 2011). TA systems are characterised according to the nature of the antitoxin component because the toxin is always a protein. In type I TA systems, the antitoxin is a RNA fragment which shows complementarity to the mRNA sequence of the toxin. These TA systems have not been implicated in virus resistance (Makarova *et al.*, 2013, Van Melderen and Saavedra De Bast, 2009). In type II systems, the antitoxin is a small and unstable protein which complexes with the toxin, so negatively auto-regulating the operon. This in turn leads to the development of an effective immune defence system against the invading foreign nucleic acid (Van Melderen and Saavedra De Bast, 2009, Wang and Wood, 2011). Type II systems are the most prevalent TA systems currently known in prokaryotic species. The toxin in these systems is normally degraded by an essential housekeeping protease, which in bacterial species is often Lon or ClpP. This degradation of the toxin is counterbalanced by the continuous production of the antitoxin. Maintenance of a stable level of the antitoxin is essential in order to reduce the detrimental effects of the toxin on the bacterial host (Van Melderen *et al.*, 1996, Roberts *et al.*, 1994, Lehnherr and Yarmolinsky, 1995, Christensen *et al.*, 2001, Christensen *et al.*, 2003, Christensen *et al.*, 2004, Cherny and Gazit,

2004, Aizenman *et al.*, 1996, Van Melderen and Saavedra De Bast, 2009, Sberro *et al.*, 2013). Type III TA systems contain RNA antitoxins but in this instance they inhibit toxin activity and functionality (Wang and Wood, 2011, Leplae *et al.*, 2011). Type IV and V TA systems have been proposed in the last 5 years; classification of these systems is based upon the mechanistic action of their respective antitoxins (Wang *et al.*, 2012, Masuda *et al.*, 2012, Makarova *et al.*, 2013).

1.6.5.5. Abortive phage infection (Abi)

Abi systems are often the last resort for bacterial cells to protect themselves against phage infection. These systems function after a phage particle has managed to successfully enter into a host cell and avoid the RM, CRISPR/Cas and TA systems (Stern and Sorek, 2011). The Abi system functions to block phage replication, which in turn leads to the premature death of the infected bacterial cell. This cell death decreases the amount of phage particles that can be released into the surrounding cellular environment which controls the levels of phage predation within an environment (Chopin *et al.*, 2005). This 'altruistic suicide' is thought to occur due to phage having corrupted an essential host cellular process (Stern and Sorek, 2011).

The ubiquitous nature of this defence system is apparent in a range of Gram negative and Gram positive bacteria including *Vibrio cholerae* (Chowdhury et al., 1989), *Bacillus subtilis* (Hemphill and Whiteley, 1975), *Lactococcus lactis* (Forde and Fitzgerald, 1999, Chopin et al., 2005), *Bacillus licheniformis* (Tran et al., 1999), *Shigella dysenteriae* (Smith *et al.*, 1969) and *Streptococcus pyogenes* (Behnke and Malke, 1978). A large amount of work on Abi systems has been undertaken in *Lactococcus lactis*; a Gram positive bacterium that is extensively

used for lactic acid production during the manufacture of fermented dairy products (Bouchard *et al.*, 2002). Some large dairy plants can process up to 10^6 litres of milk per day, which leads to the generation of 10^{18} lactococcal cells. Not surprisingly, this process is highly susceptible to infection by both lytic and temperate bacteriophages which can have dramatic effects on the fermentation process both biologically and economically (Whitehead and Cox, 1935, Chopin *et al.*, 2005). Chopin *et al* (2005) reviewed the Abi systems in *Lactococcus lactis* and it was identified that there were currently 20 known Abi systems, however, there may be more systems but the proteins have little or no homology to currently known proteins (Bouchard *et al.*, 2002).

1.7. AIMS AND OBJECTIVES

The aim of this study was to characterise the influence temperate bacteriophages of *P. aeruginosa* have on the progression of chronic lung infections. The temperate bacteriophages involved in this study were induced from *P. aeruginosa* isolates collected from a cohort of adult BR patients, paediatric CF patients and adult CF patients. This study is split into chapters which try to use different scientific methodologies in order to elucidate the effect that bacteriophages have on disease progression.

Initially, the work focused on looking at the ability of mixed phage communities to be induced from individual isolates of clinical *P. aeruginosa* and how they can infect different *P. aeruginosa* isolates. This was undertaken in order to determine whether the different clinical aetiologies had an effect on the dynamic interplay of phage-host infection profiles. Plaque purified phages were utilised in order to generate lysogenic bacteria and the lab strain, PAO1, was selected as the host

bacterium for these lysogenic conversions. The lysogens were further characterised for differences in growth rates and antibiotic resistance.

Liquid Chromatography Mass Spectroscopy (LCMS) was also utilised in order to determine the impact that phages have on host cell metabolism during pellicle formation. Metabolic profiles were compared between *P. aeruginosa* isolates which were lysogenic or non-lysogenic for prophage formation.

Due to the continuous nature of phage and bacterial interactions in the lower lung, it was decided to undertake a metagenomics study of the phages in order to determine whether there were any functionality differences that could be seen between the different aetiological subgroups. Metagenomic analysis utilising Metagenomics Rapid Annotations based on Subsystem Technology (MG–RAST) was conducted.

2. GENERAL MATERIALS AND METHODS

2.1. BACTERIAL STRAINS

This study used 94 clinical *Pseudomonas aeruginosa* (*P. aeruginosa*) bacterial isolates (10 paediatric Cystic Fibrosis (CF) isolates, 37 adult CF isolates, 16 < 10 years since clinical diagnosis bronchiectasis (BR) isolates and 30 > 10 years since clinical diagnosis BR isolates); sample ID numbers are detailed in table 2.1

These isolates were all stored post collection from the Freeman Hospital or Royal Victoria Infirmary, part of the Newcastle upon Tyne Hospital Trust, UK in 50 % volume per volume (v/v) Glycerol (ReAgent, Runcorn, UK) at -80 ^{0}C.

The samples were collected from the Freeman Hospital, Newcastle upon Tyne Hospital Trust on muller-hinton agar slopes. Prior to collecting the samples, the sputum samples were grown on blood agar, chocolate bacitracin agar and chocolate agar prior to incubation at 37 ^{0}C for 48 hours. In order to confirm that *P. aeruginosa* had in fact been isolated mass spectroscopy was carried out on the samples (personal communication).

In the CF cohort, there were 4 *P. aeruginosa* strains that were typed as the Liverpool Epidemic Strain, 4 typed as the Manchester Epidemic Strain, 2 typed as the Leeds Epidemic Strain, 1 strain typed as the Midlands-1 Epidemic Strain and 2 strains which were typed as Clone C Epidemic Strains. There were 13 mucoid *P. aeruginosa* samples in the cohort and 34 non-mucoid *P. aeruginosa*.

In the BR cohort, the only epidemic strains belonged to Clone C and there were only three strains typed in this sample set. There were also 22 mucoid *P. aeruginosa* samples and 25 non-mucoid *P. aeruginosa* samples.

Table 2.1: Sample ID for the 94 *P. aeruginosa* isolates and their clinical origin. More detailed clinical data for each sample is included in appendix data 1 and 2.

Sample ID	Clinical Origin
3, 5, 6, 16, 23, 24, 28, 30, 34, 42, 44, 52, 54, 55, 57, 60, 63, 65, 67, 72, 74, 77, 78, 79, 81, 121, 125, 126, 127, 136, 140, 142, 145, 177, 183, 211	Adult CF
47, 53, 69, 70, 124, 165, 187, 208, 213, 214	Paediatric CF
52, 53, 58, 136, 141, 143, 1, 146, 153, 161, 177, 178, 181, 195, 197, 199, 200, 201, 204, 205, 208, 213, 222, 228, 243, 244, 285, 313, 322, 326	Over 10 BR
59, 123, 133, 150, 152, 193, 206, 227, 233, 293, 298, 299, 319, 320, 327, 331, 332	Under 10 BR

2.2. CHEMICALS AND GROWTH MEDIA

2.2.1. Growth Conditions

Unless stated, liquid bacterial cultures of *P. aeruginosa* were grown in Lysogeny Broth [LB] (Scientific Laboratory Supplies, Hessle, UK) and 0.01 M $CaCl_2$ (Sigma Aldrich, Gillingham, UK). LB contains tryptone, sodium chloride, yeast extract and water.

Constituents for each of the agars and broths used in this thesis are shown in table 2.2. Bacterial cultures were propagated at 37 ℃ under aerobic conditions, shaking at 200 revolutions per minute (rpm) for 18 hours. In order to propagate the cultures, LB stock plates were grown at 37 ℃ for 18 hours in a static incubator prior to being stored at 4 ℃ for < 1 month.

Table 2.2: Constituents of all the media and broths utilised during this investigation.

Media/Agar	Constituents
1 M $CaCl_2.2H_20$	147.02 g $CaCl_2.2H_20$ 1000 mL H_20
LB Broth + 0.01 M $CaCl_2.2H_20$	12.5 g LB 500 mL H_20 5 mL 1 M $CaCl_2.2H_20$
LB Stock plates	12.5 g LB 7.5 g High Clarity Agar (Lab M, Lancashire, UK) 5 mL 1 M $CaCl_2.2H_20$/500 mL H_20 500 mL H_20
Soft Agar	5g LB 0.8 g High Clarity Agar 2 mL 1 M $CaCl_2.2H_20$ 200 mL H_20

2.2.2. Bacteria and phage storage

Unless stated, all the bacterial isolates were stored at -80 ℃ for long-term storage in 50 % glycerol. Laboratory or working stocks of bacteria were stored on LB stock plates at 4 ℃. Liquid phage stocks were also stored at 4 ℃ and checked every month for viability.

2.2.3. Sterilisation of media and glassware

To maintain pure enrichment of bacterial cultures and phage preparations, all the glassware and reagents used were autoclaved at 120 ℃ for 15 minutes at a pressure of 1.0 bar.

2.2.4. Equipment

The media used in this thesis is stated in the text whilst the equipment including the manufacturers and the associated addresses are detailed in appendix data 3.

2.3. MICROBIOLOGY AND PHAGE BIOLOGY

2.3.1. Raising microbial cultures and enumeration of bacteria

As *P. aeruginosa* carries other transposable genetic elements alongside bacteriophages, it was essential not to store working stocks of isolates together. Therefore, small batches of *P. aeruginosa* were raised 2 days prior to experimentation from -80 ℃, streaked onto individual LB plates and then cultures in 10 mL of LB media were raised from the single colonies (37 ℃, 200 rpm). To enumerate bacteria in culture, 10-fold series dilutions and serial plating

onto LB allowed a viable cell count to be calculated for each microbial culture following an 18 hour incubation period at 37 ℃.

2.3.2. Growth curves

To identify the growth profiles of the *P. aeruginosa* isolates used, the bacteria were grown for an 18 hour period and the increase in the optical density of the culture over this time period was detected on the Synergy HT plate reader. 75 µL of culture (OD_{600} 0.03) was added to an assigned well in a 96 well cell culture plate and absorbance readings were taken serially every 30 minutes for 3.5 hours, followed by readings every hour for a period of 7 hours before the final reading was taken at 18 hours. Between readings, the plates were incubated at 37 ℃. The optical density was detected using a 600 nanometer (nm) wavelength and data was recorded using Gen5 1.06.

2.3.3. Bacteriophage induction from *P. aeruginosa*

Bacteriophages were chemically induced from *P. aeruginosa* using the floroquinolone antibiotic, Norfloxacin (NFLX) (1 µg.mL $^{-1}$). NFLX (10 mg) was dissolved in H_2O with limited additions of Sodium Hydroxide (NaOH) and made up to a final volume of 10 mL; aliquots were stored at -20 ℃.

From a single colony, 18 hour cultures were raised as previously described in section 2.3.1. A 2 % (v/v) inoculum from this culture into fresh 10 mL LB offered an actively growing culture from which to induce bacteriophages. The bacterial cultures were then grown to mid exponential growth phase (0.4 - 0.6 OD_{600}). NFLX was added for 1 hour (37 ℃, 200 rpm) to stimulate cellular stress and therefore, bacteriophage induction. The culture containing NFLX was diluted

(1:10) to limit the cytotoxic effect of the drug, whilst also allowing for the cascade of phage induction to occur (Matsushiro *et al.*, 1999, Smith *et al.*, 2007, James *et al.*, 2001). Phage lysates were filtered through 0.22 µM syringe filters (Scientific Laboratory Supplies) and stored at 4 ℃ for < 1 week.

2.3.4. Bacteriophage enumeration

2.3.4.1. Spot infection assays

To determine the ability of a phage to infect a bacterial host, spot infection assays were utilised. 100 µL of mid exponential growth phase *P. aeruginosa* (0.4 - 0.6 OD_{600}) was added to 5 mL 0.4 % weight per volume (w/v) soft agar aliquots and overlaid onto solid LB plates. Once surface dry, 10 µL of phage lysate was spotted onto the soft agar containing the bacteria and incubated for 18 hours at 37 ℃. In order to determine whether the *P. aeruginosa* host was susceptible to phage infection, one would expect to see zones of clearance on the plate whereas, results like this would not be expected on non-susceptible hosts.

2.3.4.2. Purification and phage enumeration using plaque assays

Plaque assays can be used to enumerate and isolate individual phages from mixed communities and to confirm the presence or absence of pyocin production. A ten-fold serial dilution of each phage lysate was mixed with 100 µL of phage-sensitive mid-exponential *P. aeruginosa*. These dilutions were incubated for 25 minutes at 37 ℃. Each dilution was added to 5 mL of 0.4 % (w/v) soft agar and overlaid onto bottom agar plates then incubated at 37 ℃ for 18 hours (Sambrook *et al.*, 1989, Green and Sambrook, 2012). Individual plaques were picked and the

phage was allowed to disperse into 450 µL of LB broth for 18 hours at 4 ℃ (Sambrook *et al.*, 1989, Green and Sambrook, 2012). Plaque assays from these picked plaques allowed for the observation of the LB agar plate with the most discrete plaque morphologies. The agar from this plate was scraped and stored for 18 hours at 4 ℃. The agar and bacterial debris was pelleted by centrifugation at 4,000 rpm for 10 minutes, 4 ℃. The supernatant was then filtered through a 0.22 µM syringe filter. These phage preparations were then used for subsequent rounds of ten-fold serial dilutions and plaque assays to validate a pure enrichment of a single bacteriophage (if required). The Plaque forming units per mL ($Pfu.mL^{-1}$) were calculated in order to determine the amount of phage particles present in the lysate.

2.3.5. Lysogen formation

Using a ratio of phage to bacterium (MOI 0.1), it is possible to stimulate lysogenic over lytic phage infection. With an increase in multiplicity of infection (MOI), there is a drive towards lysogenic infection (Boyd, 1951, Fry, 1959, Kourilsky, 1973, Lieb, 1953). This culture was then incubated for 30 minutes at 37 ℃ and spread plated onto LB plates. These plates were incubated at 37 ℃ for 18 hours. Individual colonies were picked and patched onto LB plates, enriched and stocked in 50 % glycerol at -80 ℃. To confirm lysogeny, without a phage related genetic marker of infection, each putative lysogen was subjected to genomic DNA extraction (section 2.5.1.1.) and a DNA restriction endonucleases digestion (section 2.5.6.). Any change in the profiles may relate to the integration of phage genomic DNA into the bacterial backbone. The lysogens generated in this study were all derivatives of PAO1 (standard laboratory *P. aeruginosa* strain) with the

addition of a paediatric CF phage, an adult CF phage, a < 10 BR phage or a > 10 BR phage.

2.4. MICROBIOLOGICAL TECHNIQUES

2.4.1. Minimum Inhibitory Concentration (MIC) test

The assays were set up using an adapted protocol from (Andrews, 2001). MIC assays were set up in order to test whether there was any alteration in antibiotic tolerance between the lysogens generated previously (section 2.3.5.) and naïve PAO1. In brief, a 150 µL total volume comprising of a 1:1 ratio of *P. aeruginosa* culture at an OD_{600} of 0.03 and lysogeny broth containing the antibiotic concentration was utilised. In order to determine the MIC, a five-fold dilution for each antibiotic, listed in table 2.3 was prepared. All the antibiotics were obtained from the Freeman Hospital CF clinic, as these are antibiotics that are routinely given to the CF patients in the Newcastle upon Tyne Hospital Trust.

Colistin was used at a stock concentration of 96.29 mM, Ceftazidime was used at a stock concentration of 45.55 mM. Piperacillin was used at a stock centrationoin of 101.69 mM and Meropenem was used at a stock concentration of 137.25 mM. Colistin, Ceftazidime and Meropenem were all reconstituted in water whilst Piperacillin was reconstituted in methanol. The five-fold dilution series were prepared using LB broth.

The MIC assays were prepared in triplicate with readings taken at 3 time points (0 hours, 9 hours and 18 hours). Plates were prepared in triplicate so that there was no contamination risk upon the removal of the breathable seal before the absorbance readings were taken. Breathable seals (AeraSeal Sterile, Alpha

Labs, Eastleigh, UK) were used to allow for aerobic growth throughout the process. The plates were kept at 37 ℃ and rotated at 50 rpm between absorbance readings to maintain aeration in the cultures and to limit biofilm formation. The absorbance of the plate was detected using a 600 nm wavelength and the data was recorded using Gen5 1.06 plate reader.

In order to determine the differences in antibiotic tolerance between the naïve *P. aeruginosa* (PAO1) and the lysogens, it was decided to normalise the results according to PAO1. This gave strength to the conclusions that were drawn from this analysis.

Table 2.3: Antibiotics utilised in the MIC work including their mode of action and stockist.

Antibiotic	Target	Stockist
Ceftazidime	Peptidoglycan synthesis	Sigma Aldrich
Colistin	Cytoplasmic membrane	Forest Laboratories, London, UK and Sigma Aldrich
Meropenem	Peptidoglycan synthesis	Fresenius Kabi Limited, Runcorn, UK
Piperacillin	Peptidoglycan synthesis	Bowmed Ibisqus Limited, Wrexham, UK

2.4.2. Growth of *P. aeruginosa* as a pellicle

It was decided to use a pellicle for this thesis, as a pellicle grows at the air liquid interface thus, inferring possible clinical relevance to the results. A pellicle is a derivative of a biofilm; the bacterial cells are protected from environmental changes and stimuli by the formation of an extracellular matrix. As phages can also be isolated from environments in which these pellicles develop, a method was derived to study the development and impact of phage on pellicle formation.

6 well tissue culture plates were used for all pellicle studies; 2 mL of LB was added to each well. A 10 % inoculum of a freshly sub-cultured lysogen (OD_{600} 0.03) was added to each well and the plates were sealed and left at 37 ℃. Plates were generated in quadruplicate so that they could be removed at the specific time points required. The plates were removed when the cultures were in early growth, (3 hours), mid-exponential growth phase (5 hours) and stationary phase growth (10 hours). A final reading was taken 36 hours post inoculum.

The pellicle was separated from the fresh cultures via careful extraction using a P1000 pipette, in most instances however, there was no remaining fresh culture as it had all formed part of the pellicle structure.

2.4.3. 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carbox-anilide (XTT) assay

XTT is a tetrazolium dye that is used in quantification assays to determine viable cells in a community *via* metabolic activity (Peeters *et al.*, 2008, Gabrielson *et al.*, 2002, McCluskey *et al.*, 2005). The XTT assay indicates the metabolic activity of *P. aeruginosa* during both pellicle formation and growth. 1 mL of pellicle was sheared by pipetting and then further sheared by forcing it through a 0.22 µM syringe filter, the filter was then washed through using 1 mL of molecular grade

water (Fisher Scientific, Loughborough, UK). 80 µL of the pellicle suspension was added to the appropriate well of a 96 well plate along with 20 µL of the working concentration of XTT (Sigma Aldrich). The stock XTT was reconstituted with 5 mL filtered 1 x TAE (to generate a stock of 1 $mg.mL^{-1}$). To generate a working concentration of 0.3 $mg.mL^{-1}$, 1 mL of the reconstituted stock was added to 3 mL 1 x TAE. Readings were taken every 10 minutes over a 2 hour period to determine any metabolic changes in the pellicles over time using Gen5 1.06 (490 nm).

2.4.4. Twitching motility assays

Bacterial isolates were grown from -80 °C stocks onto solid LB plates. Following incubation, a colony was selected and pierced straight into a fresh LB plate ensuring that the sterile loop containing the bacterial culture touched the base of the plate (Winstanley *et al.*, 2005, Fonseca *et al.*, 2004, Semmler *et al.*, 1999). LESB58 was used as a negative control during these assays as it is known that this *P. aeruginosa* isolate is non-motile (Winstanley *et al.*, 2009). PAO1 was used as a positive control.

When looking at the effect of phages on bacterial motility, 100 µL of mixed phage lysate was spotted onto the plate in the same location as the bacterial colony. The plates were then incubated at 37 °C for 18 hours before the agar was carefully removed. The empty plates were flooded with 1 mL of crystal violet (TCS Biosciences, Botolph Claydon, UK) and the staining was allowed to progress at room temperature (~ 16 °C) for 30 minutes. Crystal violet was used as it binds to negatively charged molecules such as DNA thus, indicating where the bacterial cells have moved during the twitching assay. After incubation the stain was washed off and bacterial motility was recorded in millimetres (mm).

2.5. MOLECULAR TECHNIQUES

2.5.1. Phage DNA Isolation method development

After induction and filtration in section 2.3.3.; bacterial chromosomal DNA will remain within the phage lysate. Bacterial chromosomal DNA was removed using 1 µL of TURBO DNAse and 1 µL of RNAse Cocktail (Life Technologies Limited, Paisley, UK) and incubation at 37 ℃ for 30 minutes. This was followed by heat inactivation at 65 ℃ for 10 minutes along with the addition of 30 µL 0.5 M EDTA, shown in table 2.9. The manufacture's guidelines were followed for this part of the thesis.

2.5.1.1. Phage precipitation using polyethylene glycol (PEG8000)

PEG8000 (Promega, Southampton, UK) purification was utilised in order to aid the purification process through the precipitation of phages, the protocol described by Sambrook *et al* (1989) was used. 0.5 M final concentration of NaCl (added in order to change the weight of the phage particles) was added to the NFLX induced phage lysate at 4 ℃. The lysates were then centrifuged at 7,000 rpm for 15 minutes, 4 ℃ in order to pellet the bacterial debris. The supernatant was carefully removed and used for further downstream processing and the pellet containing the bacterial debris was discarded. 10 % final concentration of PEG8000 (w/v) was added slowly to the supernatant prior to being left overnight (18 hours) at 4 ℃. This culture was then centrifuged at 10,000 rpm for 20 minutes at 4 ℃. In this instance, the supernatant was discarded and the pellet containing the phage particles was retained. The speed of the centrifugation process allowed for the phage to become pelleted as PEG8000 altered the

charge and weight of the particles in the lysate thus, causing the phage to pellet. To the pellet, 7.5 mL TBT buffer (100 mM Tris-HCl (pH 7.0), 100 mM NaCl, 100 mM $MgCl_2$, pH 7.0) was added along with an equivalent volume of Chloroform (Fisher Scientific). The sample was then centrifuged again at 4,000 rpm for 15 minutes at 4 °C. 1 mL of the top phase was removed and used for further downstream processing analysis.

2.5.1.2. QIAGEN QIAmp MinElute Viral Spin Kits

QIAGEN QIAmp MinElute Viral Spin Kits (QIAGEN, Manchester, UK) were also used in order to purify the phage nucleic acid; this kit can purify DNA greater than 200 bp. The manufactures' guidelines were followed for this kit.

25 µL of QIAGEN protease was added to a 1.5 mL microcentrifuge tube, followed by 200 µL of the phage lysate. 200 µL of Buffer AL/RNA-AVE was also added. An incubation period of 15 minutes at 56 °C followed, prior to 250 µL of absolute ethanol being added. The lysate was then incubated for 5 minutes at room temperature (~ 16 °C). All the lysate was loaded onto the provided QIAmp MinElute column. The column was centrifuged at 8,000 rpm for 1 minute and the collection tube and filtrate discarded, the column was then reassembled with a new collection tube. 500 µL of Buffer AW1 was then loaded onto the column and the column was centrifuged at 8,000 rpm for 1 minute and again the collection tube containing the filtrate was discarded (Buffer AW1 denatured the proteins in the sample and allowed them to pass through the column). This process was repeated when 500 µL of Buffer AW2 was loaded onto the column and again when 500 µL of absolute ethanol was loaded (Buffer AW2 removes salts from the column thus, aiding DNA purification). In order to prevent any problems with downstream applications due to ethanol carryover, the column was dried by

centrifugation at 14,000 rpm for 3 minutes. 75 µL of Buffer AVE (warmed to 37 ℃ to increase elution efficiency) was added to the column, which was placed in a new collection tube before centrifugation at 14,000 rpm for 1 minute. The filtrate was retained and used for further downstream processing.

2.5.1.3. Chosen method: NORGEN phage DNA isolation kit

The NORGEN phage DNA isolation kit (Geneflow Limited, Lichfield, UK) was the method chosen to purify the viral DNA in this thesis. The kit works via affinity binding and has no DNA size prerequisite which makes it an ideal kit for *de novo* work. The manufactures' guidelines were followed for this kit but in the brief the protocol is as follows. 1 mL of phage lysate was transferred to a 15 mL sterile falcon tube along with 500 µL of lysis solution prior to a vigorous vortex for 10 seconds. The falcon tube containing the phage and lysis buffer was incubated at 65 ℃ for 15 minutes; the tube was inverted every 5 minutes to ensure efficient chemical mixing and heat lysis. 320 µL of isopropanol (Thermo Fisher Scientific) was added to the falcon tube and briefly vortexed. DNA is less soluble in isopropanol meaning that DNA precipitates out of solution rapidly and at lower concentrations thus, decreasing the amount of time required for sample purification. 650 µL of lysate was added to a provided spin column prior to centrifugation at 8,000 rpm for 1 minute, the filtrate containing the cell debris and unwanted protein was discarded. This step was repeated until all the phage lysate in the 15 mL falcon tube had been run through the spin column so that the entire DNA load was bound to the matrix of the column. 400 µL of wash solution was added to the column and the column was again centrifuged at 8,000 rpm for 1 minute and the flow through was discarded. This wash step was undertaken in triplicate in order to ensure efficient washing and the removal of any cell debris or protein contamination. Ethanol carryover can have a detrimental effect in

downstream DNA analysis so the column was centrifuged at 14,000 rpm for 2 minutes in order to dry the resin and remove any excess ethanol. The column was then placed into a fresh collection tube to which 75 µL of elution buffer (warmed to 37 ℃ prior to use in order to increase efficiency) was added. The column was again centrifuged at 8,000 rpm for 1 minute. The flow through from this centrifugation was then collected and run through the column again in order to increase the yield of the final phage DNA sample.

2.5.2. Bacterial DNA Isolation

P. aeruginosa DNA was isolated using the UltraClean Microbial DNA isolation kit (Mo Bio, Cambio, Cambridge, UK); this kit yields DNA that is greater in size than 50 Kb and each filter in the kit has the capacity to bind 20 µg of DNA.

The manufactures' guidelines were followed for this kit. In brief, 1.8 mL of mid exponential *P. aeruginosa* culture was added to a 2 mL collection tube and centrifuged at 10,000 rpm for 30 seconds to pellet the bacterial cells. The supernatant was discarded before the tubes were centrifuged at 10,000 rpm for 30 seconds. The cell pellet generated from the previous two centrifugation steps was re-suspended in 300 µL of microbead solution prior to the sample being transferred to a microbead tube. The microbead solution stabilises the sample as well as homogenously dispersing the cells in order to allow for efficient lysis. 50 µL of solution MD1 was added to the microbead tube; this solution contains sodium dodecyl sulphate (SDS) which allows for cell lysis and solubilisation of membrane lipids. The microbead tubes were vortexed at full speed for 10 minutes in order to allow for chemical and mechanical lysis. The tubes were centrifuged at 10,000 rpm for 30 seconds in order to pellet the cellular debris and the supernatant retained. 100 µL of MD2 was added to the collection tube which

contained the harvested supernatant prior to incubation at 4 ℃ for 5 minutes. MD2 aids the precipitation of non-DNA organic and inorganic material which can potentially have a negative effect in downstream applications. The incubated sample was centrifuged at 10,000 rpm for 1 minute prior to the addition of 900 µL of MD3. MD3 has a high salt concentration and generates the gradient required to bind DNA to the spin filter membrane. 700 µL of the supernatant (containing both the lysate and buffer MD3) was added to a tube containing the spin filter and centrifuged at 10,000 rpm for 30 seconds. The flow through was discarded and the remaining 700 µL of the sample and MD3 was run through the column. These wash steps ensure that the DNA remains bound to the column whilst the contaminants are washed through the column and discarded. 300 µL of MD4 was added to the spin filter (MD4 is an ethanol based wash solution which cleans the DNA sample). The sample was then centrifuged at 10,000 rpm for 30 seconds and the flow through discarded. In order to dry the membrane and remove final traces of MD4, the column was centrifuged at 10,000 rpm for 1 minute. 50 µL of warmed MD5 elution buffer was added to the centre of the membrane and allowed for the elution of DNA from the column.

2.5.3. NanoDrop 1000 Spectrophotometer V 3.7

The nanodrop is a UV spectrophotometer that utilises small volumes of a DNA/RNA sample in order to determine the purity and quantity of the nucleic acid. After the blank reading (1 µL of elution buffer appropriate to the sample in question) the stage was cleaned with water and 1 µL of the sample was loaded in order to determine the concentration and nucleic acid yield.

The 260/280 nm ratio assess the purity of the DNA/RNA in the sample; a ratio of around 1.8 can be used to assess whether the sample contains pure DNA and a

ratio of around 2.0 can be used to assess whether the sample contains pure RNA. When the ratio is appreciably lower in both instances, then it indicates the presence of a contaminant which has absorbance of or near 280 nm. Commonly the contaminant is either a protein or phenol which is left over from the nucleic acid preparation stage.

The 260/230 nm ratio assess the purity of nucleic acid in the sample; an accepted purity ratio falls between 2.0 and 2.2. If the ratio is lower than expected, it is possible that there is contamination with a product that has an absorbance of or near 230 nm. Commonly these contaminants can be EDTA, carbohydrates and again phenol and these are again often leftovers from DNA prep kits.

2.5.4. QUBIT 2.0

In order to effectively test the DNA concentration prior to sequencing, the QUBIT 2.0 (Life Technologies Ltd, Paisley, UK) was used. The QUBIT 2.0 fluorometer is a bench-top fluorometer that is highly sensitive and utilises fluorescence based techniques in order to quantify DNA, RNA and protein. The QUBIT dsDNA high sensitivity (HS) assay kit was used for this analysis (Life Technologies Ltd). 197 µL of HS buffer was combined with 1 µL of fluorescence dye and 2 µL of DNA. This occurs in minimal light as the reagents are light-sensitive and the reagents are also temperate-sensitive so handling is kept to a minimum. The tubes were vortexed for 5 seconds prior to incubation in the dark for 2 minutes, in order to let any bubbles generated by vortexing settle. The sample can then be read on the fluorometer selecting the dsDNA option; care was taken to observe for bubbles and scratches on the tube which may influence the output reading.

2.5.5. Polymerase Chain Reaction (PCR)

All the primer sequences used in this investigation are detailed in table 2.4. Regardless of the primers and their concentrations all the PCR's were set up according to table 2.5, with 25 µL reactions being used throughout this work. The timings for each individual PCR experiment varied according to the primers used and their associated annealing temperatures. The various time alterations for each primer set are described in table 2.6.

Table 2.4: Details all the primer sequences that were used during this investigation at a 1 x working concentration. All the primers are purchased from Integrated DNA Technologies, Heverlee, Belgium.

Name	Sequence (5' – 3')	Region	Reference
V3F	CCTACGGGAGGCAGCAG	V3 region of the 16S rRNA	(Muyzer *et al.*, 1993)
V3R	ATTACCGCGGCTGG	V3 region of the 16S rRNA	(Muyzer *et al.*, 1993)
Q 5'	ATGTTCTTATGGTTCACCG	*Q* gene of $\phi 24_B$	(Smith *et al.*, 2007)
Q 3'	TTACGATCGTAAACTATTTTT	*Q* gene of $\phi 24_B$	(Smith *et al.*, 2007)
tRNA thr	CGAATGAGCTGCTCTACCGACAGAGCT	*pilA*	(Kus *et al.*, 2004)
pilA	TCCAGCAGCATCTTGTTGACGAA	*pilA*	(Kus *et al.*, 2004)

Table 2.5: PCR set up; all the suppliers and volumes of reagents used are listed.

PCR Component	Amount	Supplier
Phusion High Fidelity PCR Mastermix with high fidelity (HF) Buffer	12.5 µL	New England Biolabs, Hitchin, UK
Forwards Primer (10 mM)	1.25 µL	Integrated DNA Technologies
Reverse Primer (10 mM)	1.25 µL	Integrated DNA Technologies
DNA	1 µL	Own
Biotech grade water	Make up to 25 µL	Fisher Scientific

Table 2.6: PCR timings utilised throughout this thesis.

	Temperature	Timings	Stage	Cycles	Reference
16 S rRNA	98 ℃	10 minutes	Initial Denaturation		(Muyzer *et al.*, 1993)
	98 ℃	1 minute	Denaturation	30 cycles Drop 0.5 ℃ / cycle	
	55.5 ℃	30 seconds	Annealing		
	72 ℃	1 minute	Extension		
	72 ℃	7 minutes	Final Extension		
	4 ℃	Forever	Hold		
***Q* gene**	98 ℃	10 minutes	Initial Denaturation		(Smith *et al.*, 2007)
	98 ℃	1 minute	Denaturation	30	
	55.5 ℃	30 seconds	Annealing		
	72 ℃	1 minute	Extension		
	72 ℃	7 minutes	Final Extension		
	4 ℃	Forever	Hold		
***pilA* gene**	95 ℃	5 minutes	Initial Denaturation		(Kus *et al.*, 2004)
	95 ℃	30 seconds	Denaturation	30	
	45 ℃	60 seconds	Annealing		
	72 ℃	2 minutes	Extension		
	72 ℃	7 minutes	Final Extension		
	4 ℃	Forever	Hold		

2.5.6. Restriction Digestion

In order to differentiate between the plaque purified phages (section 2.3.4.2.), they were subjected to restriction digestion in order to determine any genetic differences between the phages. The pure phage DNA (section 2.5.1.3) was used at 300 ng and combined with 5 units of each restriction digest enzyme; BamHI, ClaI, EcoRI – HF and HindIII (New England Biolabs), cut sites are shown in table 2.7. 1.5 µL of Cut Smart buffer (New England Biolabs) was used and the volume made up to 15 µL with sterile water (Fisher Scientific). The digests were left at 37 ℃ for one hour in order to allow for effective cleavage but the time was restricted in order to prevent star activity. Post digestion, the products were run on 1 % agarose gels (section 2.5.7). The setup of the digestions is shown in table 2.8.

Table 2.7: Restriction digest cleavage sites shown in both the 5' to 3' orientation and in the 3' to 5' orientation, taken from the New England Bio labs website (accessed 20.07.15). HF refers to high fidelity enzymes which were used wherever possible.

Enzyme	Cut sites
BamHI – HF	5'…GˇGATCC…3' 3'…CCTAGˆG…5'
ClaI	5'…ATˇCGAT…3 3'…TAGCˆTA…5''
EcoRI - HF	5'…GˇAATTC…3' 3'…CTTAAˆG…5'
HindIII	5'…AˇAGCTT…3' 3'…TTCGAˆA…5'

Table 2.8: Volumes of reagents used to set-up the restriction digestion reactions. The restriction digestion enzymes are used at a volume of 0.5 µL and the final reaction volume is 15 µL.

	ΦCF70	**ΦCF24**	**ΦBR123**	**ΦBR136**
Water (µL)	8.5	10.5	11	10.5
Smart Cut Buffer (µL)	1.5	1.5	1.5	1.5
DNA (µL)	3	1	0.5	1
Restriction Digest Enzyme (total µL)	2.0	2.0	2.0	2.0

2.5.7. Agarose Gel Electrophoresis

In order to visualise the PCR products, 1 % agarose gels were used, the gel setup is detailed in table 2.9. The agarose (Melford Laboratories Limited) and 1 X Tris-acetate-EDTA (TAE) was heated to approximately 50 ℃ in order to melt the agarose. 5 µL of SYBR safe DNA stain (Life Technologies Ltd) was added to the gel in order to aid visualisation of the DNA and the mixture was poured into gel-forming casts (two sizes available depending on sample set size) prior to a comb being inserted to generate the wells required. The gel was allowed to set for around 30 minutes prior to the gel being immersed in 1 x TAE, the sample wells and electrodes needed to be covered with TAE. The wells on either side of the gel were loaded with 5 µL of HyperLadder 1 Kb (Bioline, London, UK) and 5 µL of Quick-Load 100 bp ladder, respectively (New England Biolabs). The DNA samples were mixed with 5 µL of loading dye (Bioline) and loaded into the appropriate wells. The loading dye contains a pre mixed loading buffer of two dyes; Dye 1 (pink/red loading front) and Dye 2 (blue loading front). The gels were then run at 120 milliamps (mA) for 25/40 minutes depending on gel size. Results were viewed under ultraviolet (UV) transillumination.

Table 2.9: Constituents for the generation of Agarose Gels and the associated buffers required for gel electrophoresis.

Media/Gel	Constituents
0.5 M Ethylenediaminetetraacetic acid (EDTA)	93 g EDTA (Melford Laboratories Limited, Ipswich, UK) 500 mL H_20 Slowly dissolve with NaOH to generate a pH of 8
50 x TAE (Tris-acetate-EDTA)	242 g Tris 57.1 mL Acetic Acid 100 mL 0.5 M EDTA Make up to 1 L with H_20
1 x TAE	20 mL 50 x TAE 980 mL H_20
1 % Agarose Gels	0.3 g Agarose/30 mL 1 x TAE 1 g Agarose/100 mL 1 x TAE

2.6. ELECTRON MICROSCOPY

Electron microscopy was undertaken at the Electron Microscopy Unit, Newcastle University, Newcastle upon Tyne, UK. In brief, a 5 – 10 µL droplet of the biological sample was added to the support film of a mesh grid (Gilder Grids, Grantham, UK). After a few seconds, the droplet was touch dried using filter paper to leave behind a thin film on the grid which contained both the absorbed and freely suspended biological sample. 10 µL of 2 % aqueous uranyl acetate (Agar Scientific, Stansted, UK) was added to the grid before being touched dried. Uranyl acetate was used as the negative stain and it allowed for quick staining and visualisation of the biological sample. The mesh grid was allowed to completely air dry prior to imaging. The grids were examined using a Philips CM 100 Compustage (FEI, Oregon, USA) Transmission Electron Microscope (Philips UK, Guildford, UK). The biological sample was visualised using a 100 kV current and the digital images generated were collected using an AMT CCD Camera (Deben, Bury St Edmunds, UK).

2.7. LIQUID CHROMATOGRAPHY – MASS SPECTROSCOPY (LCMS)

LCMS was used to study the metabolite profiles of the bacteria both with and without phage infection. 1 mL of harvested pellicle (section 2.4.2) was added to a sterile 1.5 mL eppendorf and washed with 1 x phosphate-buffered saline (PBS) (Sigma Aldrich) prior to centrifugation at 14,000 rpm for 20 minutes. This procedure was repeated in triplicate in order to clean the pellicle as effectively and efficiently as possible. The eppendorf lid was pierced with a flamed needle and the tube was placed in the - 80 ℃ freezer for a minimum of 30 minutes in order to completely freeze the pellicle samples prior to lyophilisation. The samples were then placed in the freeze drier for 18 hours at temperatures reaching -55 ℃ (Alpha 1 – 2, Scientific Laboratory Supplies).

The lyophilised products were stored at - 80 ℃. When required, the samples were weighed to 0.001 g (+/- 0.0005 g) prior to the addition of LCMS Grade Methanol (Sigma Aldrich) and 0.66 % LCMS Grade Formic Acid (Thermo Fisher Scientific Ltd). A stock solution of methanol and formic acid was created and the volume added to each sample depended on the lyophilised product weight, this method was utilised in order to generate a normalised 0.5 % (w/v) solution. The samples were vortexed for 3 minutes prior to sonication (Bandelin Sonorex, Sigma Aldrich) for 75 minutes at 25 ℃. The samples were cooled on ice every 15 minutes to prevent pressure build up. The resulting product was filtered through 0.22 µM syringe filters (Camlab, Cambridge, UK) and added to LCMS vials containing 200 µL inserts (Fisher Scientific Limited). The UltiMate 3000 LC System [C18 Thermo Accurate column (Thermo Scientific)] and Q Exactive (Thermo Scientific) was used. Acetonitrile (VWR, Lutterworth, UK) and water (VWR) were used as the mobile phases.

2.7.1. Run parameters LCMS

The experimental work was undertaken in triplicate as well as each sample having a technical duplicate. All the samples were loaded onto the LCMS in a random order (Excel function = rand(), sort smallest to largest). Between every 9 samples, a blank sample (0.5 % TFA [Trifluroacetic acid, Sigma Aldrich] in 99.5 % LCMS Grade Water [VWR]) was run alongside a pooled sample, this allowed for maintenance of the columns accuracy and performance. The injection time for each of the samples was 25 minutes and the gradient was 17 minutes to allow for optimal levels of acetonitrile (VWR) and water (VWR) containing 0.1 % formic acid (Thermo Fisher Scientific Ltd) to be achieved. 3 µL of each sample was injected onto the column along with 195 µL of acetonitrile and water; the injection

and retention time on the column determined the interaction strength between the mobile and stationary phases.

2.7.2. Analysis of metabolite profiling Progenesis QI

The raw data files (MZNLD files) were downloaded from the Q Exactive post run, in order to upload the files onto Progenesis QI 64 bit v 4.1 (Nonlinear Dynamics Limited, Newcastle Upon Tyne, UK). Thermo (.raw) data files were uploaded in an order relating to the experimental design. The files were automatically aligned and various adducts were included for both the Electrospray ionisation (ESI) positive and ESI negative runs; the adducts included are shown in table 2.10. Upon alignment, the groups of data were stratified according to the experimental design; this allowed for comparison between both growth stages and pellicles with/without phage infection. The parameters used for peak picking were stringent in order to generate the best quality data; ions released in the first 0.5 minutes of the run were excluded from further analysis as were ions released after 16.30 minutes. This was done in order to prevent any debris from the column being present in further downstream analysis.

Various search engines were used to analyse the raw data files, these include the human metabolome database (HMDB) (Wishart *et al.*, 2013, Wishart *et al.*, 2009, Wishart *et al.*, 2007) and the *E. coli* metabolome database (ECMDB) (Guo *et al.*, 2013). All the files were used in the .sdf format. The following parameters were applied to the comparison searches to aid the accuracy of these MS1 identifications. These parameters were 'mass within 0.1 Dalton (Da)', and 'fragment search theoretical and fragmentation: 12 parts per million (ppm)'. The files were then tagged in order to allow for further downstream analysis. The samples were tagged according to statistical significance including *p* values and

minimum coefficient of variation (CV) which illustrates the high degree of repeatability. Files with *p* values ≤ 0.05 and CV values ≤ 5 were tagged and combined in order to determine metabolites of interest in the raw data set. All the compounds that were identified were exported into Excel and searched against the raw data from the individual runs. Compounds with retention time differences within +/- 0.6 and a mass to charge ratio (m/z) ratio +/- 0.6 of the searched compounds were included for further analysis.

Upon further analysis, the metabolites that were significant according to a variance of importance plot (VIP) were again analysed on Progenesis in order to potentially generate a functional ID for the compound. Only metabolites with the same m/z ratio and a mass error of +/- 2 were selected for further processing but MS/MS analysis would be needed to allow for accurate identifications.

Table 2.10: Adducts included in Progenesis QI Analysis for both the Positive and Negative runs.

ESI Positive	ESI Negative
M + Na	M – H
M + K	M + FA – H
M + ACN + Na	2M – H

2.8. NEXT GENERATION SEQUENCING

The Illumina Nextera XT (Illumina, Saffron Waldon, UK) library preparation kit was used to prepare and multiplex the isolated phage DNA in order to undertake next generation sequencing (NGS) on the Illumina MiSeq platform. A 2 x 250 cycle V2 kit was used. The DNA samples were diluted to 0.2 µg/µL (Qubit 2.0 DS HS DNA Kit [Life Technologies Ltd]) (section 2.5.4.) prior to normalisation and pooling. The samples were sequenced using the NU-OMICS facility at Northumbria University. Paired end sequencing reads were provided as FASTQ files (NU-OMICS, Northumbria University at Newcastle, UK) and then subjected to downstream analysis (Tariq *et al*., 2015). The preparation process in brief, follows these basic guidelines:

- DNA is diluted to 0.2 ng/uL and tested on the QUBIT 2.0
- Amplification tagmentation occurs (two step PCR process)
- Clean up of the DNA solution via beads (removes primer dimer etc)
- Bioanalyser run undertaken (gives a general DNA fragment size and provides a rough idea on the DNA concentration of the sample)
- Normalisation step via beads (balances the DNA concentration between the samples so can generate the correct final concentration)
- Pool all the samples and add the hybridisation buffer
- Heat shock the samples so they become single stranded
- Spike the DNA prep with phix (phage polymerase)
- Load onto the cartridge (contains all the reaction mixture pre-loaded)

2.8.1. Sequencing by synthesis

Illumina sequencing utilises a sequencing-by-synthesis methodology. This involves the utilisation of single stranded DNA fragments bound to flow cells via

adaptor regions. Isothermal bridge amplification of the template strain commences upon addition of DNA polymerase (Bentley *et al.*, 2008). Post amplification, the template strand was washed away leaving the double strand DNA complex denatured thus, allowing for both strands to undergo further rounds of isothermal bridge amplification forming a DNA cluster on the flow cell (Bentley *et al.*, 2008). A universal primer bound to the template DNA strand (reverse strand was cleaved and removed) and along with fluorescently labelled terminator nucleotides allowed for the generation of thousands of reads of data. The incorporation of a terminator nucleotide sequence ensures that the process occurs in a step-wise manner. The fluorescent signals emitted upon terminator incorporation can be clearly determined by two internal detection lasers (Bentley *et al.*, 2008).

2.9. DATA ANALYSIS

2.9.1. Metagenomics Rapid Annotations based on Subsystem Technology (MG-RAST) analysis

Kyoto Encyclopaedia of Genes and Genomes (KEGG) atlas maps were generated using the tab on Metagenomics Rapid Annotations based on Subsystem Technology (MG-RAST); the default settings were used (60 % similarity of 15 amino acids).

2.9.2. Statistical analysis

2.9.2.1. Simca P analysis

The generation of data modelling plots [Principle Least Squares Discrimination Analysis (PLS-DA)] was undertaken using the SIMCA-P v 13.0 software (Umetrics, Umea, Sweden). The data was analysed according to the experimental conditions imposed upon it whilst the R^2Y value was used to assess the strength of the model and its prediction power when the data was uploaded to the software in a semi supervised manner (some information about the data was known prior to analysis). The confidence in the data was assessed using the Hotelling's *T2* tolerance limit (0.95).

2.9.2.2. ANOVA analysis

This work was undertaken in collaboration with Professor Michael Brockhurst, York University, York, UK. ANOVA analysis was utilised in order to determine the interactions between the bacteria and phages, bipartite infection networks were generated. The bacterial and phage interactions both within and between the diseases were assessed (CF phage vs. CF *P. aeruginosa*, CF phage vs. BR *P. aeruginosa*, BR phage vs. CF *P. aeruginosa*, & BR phage vs. BR *P. aeruginosa*). Nestedness was measured using the binary matrix nestedness temperature calculator (binmatnest) (Rodríguez-Gironés and Santamaría, 2006) within the 'nestedness' metric from the R package 'bipartite' - v 2.04 (Dormann *et al.*, 2008, Dormann *et al.*, 2009). All the binmatnest values given were statistically significant compared to the associated null model analysis. Network visualisations were generated using R package 'bipartite' - v 2.04.

3. CHARACTERISING THE DYNAMIC INTERPLAY AND HOST-RANGE OF TEMPERATE PHAGES INDUCED FROM *PSEUDOMONAS AERUGINOSA* COLONISING THE CHRONIC LUNG

3.1. INTRODUCTION

The dynamic interplay between phages and their bacterial hosts occurs in both environmental and laboratory settings. In the oceans, it is thought that there are 10^{23} new phage infections every second (Suttle, 2007), so it may be pertinent to think that these frequencies could be higher within the constrained environment of the lower lung. Davies *et al*. (2016) showed that in a clinically relevant environment such as the lung, the inducible phages from the Liverpool Epidemic strain (LES) were likely to have key roles in the spread of LES around a particular environment. This potentially indicates that phage infections are occurring at an elevated rate within the lung. However, lysogenic conversions of the bacterial species by the LES phages occurred at lower levels than *in vitro,* so proposing that lysogenic conversion maybe impeded within the chronic lung environment.

The observation that phages may be aiding the spread of a *Pseudomonas aeruginosa* (*P. aeruginosa*) isolate in the lung lower lung is also supported by previous work undertaken using insect and observational models (Burns *et al.*, 2015, James *et al.*, 2015, Davies *et al.*, 2016a). James *et al* (2015) showed that the presence of free temperate phage in the sputum of CF patients was comparable to the number of *P. aeruginosa* particles present; it was also shown that these phages were active *in vivo*. All these studies indicate that activity of the phage communities present within the lung.

This bacteria-phage interplay may in part be responsible for the successful colonisation of *P. aeruginosa* and how a particular clone can become the predominant strain and cause severe infections in chronic respiratory disease patients.

As described in chapter 1, *P. aeruginosa* has evolved to present certain adaptive traits and phenotypes which permit its long-term colonisation in the lower lung (section 1.4.). An example includes the generation of a mucoid phenotype which is associated with long term and chronic colonisation of the lung (section 1.5.1.2.). Mucoid strains of *P. aeruginosa* were first cultivated in 1927 from a patient suffering with an abscess of the gall bladder (Sonnenschein, 1927, Govan and Deretic, 1996) but it was not until the 1960's, that the first association was drawn between the mucoid phenotype and chronic colonisation of the lungs (Doggett, 1969, Doggett *et al.*, 1966, Doggett *et al.*, 1964, Govan and Deretic, 1996). The mucoid phenotype is caused by the overexpression of alginate through mutation and dis-regulation of the *mucA* transcriptional regulator (Speert *et al.*, 1990, Mathee *et al.*, 1999, Lee *et al.*, 2005b, Govan and Deretic, 1996).

This thesis focuses on *P. aeruginosa* isolated from cystic fibrosis (CF) and bronchiectasis (BR) patients to try to understand how these bacteria evolve within the lower lung. Only a limited number of studies look at the bacteriophages that infect and integrate into the chromosome of *P. aeruginosa* and determine how these phages affect *P. aeruginosa* adaptation, survival and evolution in the lung (Davies *et al.*, 2016a, James *et al.*, 2015). Phages are known to co-evolve and interact with their bacterial hosts. This war of infection and the subsequent exclusion occurs over a period of time and leads to the development of an 'arms-race' between the two entities.

Phages are of great interest in both academic and clinical communities due to their ability to convert their bacterial hosts, which may aid bacterial survival or drive bacterial evolution which may support their persistence in the chronic lung.

3.1.1 The role of temperate bacteriophage in bacterial adaptation and evolution

3.1.1.1. Bacteriophage superinfection

In 1957, it was determined that an isogenic phage could not infect a bacterial host if the host already harboured the same phage and this led to the generation of resistance towards the super-infecting phages (Kaiser and Jacob, 1957, Ptashne, 1967). The classical system of λ phage lysogeny maintenance involves the gene product CI (section 1.6.2.). Kaiser and Jacob (1957) found that this gene product was also responsible for preventing infection by other phages belonging to the same immunity group through inhibiting early gene expression in the invading phage.

When undertaking phage induction studies in *Shigella,* it was observed that there were 3 immunologically discrete prophages (Bertani, 1951, Bertani, 2004). These temperate phages were classified as P1, P2 and P3 (Bertani and Nice, 1954). Bacteriophage P2 further demonstrated the superinfection inhibition described by Kaiser and Jacob, in this instance the incoming phage DNA was not degraded by the host *Shigella* cell but instead was prevented from replicating alongside the primary infecting P2 phage. Copies of the invading phage were passed onto daughter cells but were unable to replicate unless the resident prophage was lost or a rare double lysogen was established (Bertani, 2004, Bertani, 1953, Bertani, 1954, Bertani, 1956).

3.1.1.2. Phage-encoded exclusion of superinfection

The prophage ϕ80 prevents superinfection within its *Escherichia coli* (*E. coli*) host cell by the expression of the Cor protein. This prophage incorporation inactivates FhuA, an *E. coli* cell surface receptor, which in turn prevents further binding at the cell surface by ϕ80 (Uc-Mass *et al.*, 2004). When this receptor is blocked, bacteriophages T1, T5, HK022, N1 also cannot bind to this receptor nor can the toxin, Colicin M (Harkness and Ölschläger, 1991, Hernández-Sánchez *et al.*, 2008). The *Salmonella* phage P22 prevents superinfection of its host cell through the expression of an inner membrane protein, SieA. SieA encodes an inner membrane protein that blocks the movement of invading DNA into the cytoplasm of the host cell, potentially through interactions with components in the DNA transport system of *Salmonella* (Susskind *et al.*, 1974, Taneja and Chakravorty, 1978). This protein prevents further infection by phage ϕL, MG178 and MG40 as well as P22 (Susskind *et al.*, 1974, Hofer *et al.*, 1995). Another mechanistic action of phage immunity is seen in *E. coli* isolates due to the expression of an 83-residue lipophilic polypeptide; the immunity protein (Imm) (Lu and Henning, 1989, Lu *et al.*, 1993). The imm protein of *E.coli* upon infection by ϕT4 or any other T-even phages, can prevent further phage infections as the DNA of the secondary invading phage is excluded from the cytosol of the host cell thus, preventing replication of the invading phage (Anderson and Eigner, 1971, Dulbecco, 1952, Graham, 1953, Lu *et al.*, 1993).

HK97, a lambdoid like phage capable of infecting *E. coli,* encodes for a protein, gp15, which is present on the phage's tail and provides resistance to the *E. coli* host from infection by other HK97 phage particles and its close relative, phage HK75. It is believed that this resistance is due to gp15 containing a putative helical transmembrane domain that is likely to localise to the inner membrane thus, preventing the entry of invading phage DNA into the cytoplasm (Cumby *et*

al., 2012). Due to the presence of this putative membrane spanning protein, when *E. coli* harbours the HK97 prophage, it is possible to classify gp15 as a member of the superinfection exclusion family of proteins/Sie (Labrie *et al*., 2010a). These proteins are classed as being expressed from phage accessory genes as they are not required for essential bacterial growth but they do provide a selective advantage for the bacterium under certain extracellular pressure and stresses.

3.1.1.3. Alteration of cell function through bacteriophage conversion

Infection and integration into the bacterial chromosome changes the nomenclature of the bacteriophage to prophage. Detailed description regarding the formation of prophages and their prevalence has been described in chapter 1 (section 1.6.4). Phage encoded diversity and the expression of accessory genes through phage integration has often been shown to offer a selective advantage to both the phage and the bacterium. An example of beneficial lysogeny is demonstrated in *Shigella* when integration interrupts the *cad*A gene, an anti-virulence gene that normally encodes for the lysine decarboxylase enzyme. This enzyme normally produces cadaverine, which is involved in blocking the action of enterotoxins. So prophage incorporation into *Shigella* can enhance the pathogencity of the lysogen (Day *et al*., 2001).

Phage incorporation can also have a negative effect on the bacterial host cell dependant on the environmental conditions, this is termed negative phage conversion [term coined by Brüssow *et al*. (2004)]. Negative lysogeny indicates that phage genes can inactivate the bacteria's chromosomal genes upon incorporation resulting in loss of cellular function which is detrimental to the bacterium (Brüssow *et al*., 2004). Negative conversion has been seen in

Staphylococcus aureus (*S. aureus*) as infection and conversion by ϕ13, a serotype F bacteriophage, inactivates the genes encoding for β haemolysin and lipase. Both these genes are involved in bacterial virulence and thus, the impact of pathogenicity is reduced (Coleman *et al.*, 1989, Lee and Iandolo, 1985).

However, lysogeny can be perceived to always be beneficial to the bacterial host cell as the phage is providing the bacteria with additional diversity compared to a naïve strain. Negative lysogeny can be classed as beneficial in some instances, such as aiding survival within a particular environment when virulence is no longer needed as an essential resource.

3.1.1.4. Moron/accessory genes encoded by bacteriophages can alter disease progression

Phages can aid the spread of bacterial disease and alter its progression. 'Lysogenic conversion' of a bacterial cell by a phage and the expression of moron or accessory genes including toxins can raise the pathogenicity of a bacterium. Moron genes, as described by Brüssow *et al* (2004) are classed as extra DNA sections that are present in some bacterial hosts upon prophage incorporation but are not required for essential bacterial growth.

Table 3.1 lists moron genes which have a direct impact on the pathogenicity of their bacterial hosts. Genetic relationship studies to identify moron genes were first undertaken in 1971 by Simon and colleagues; they utilised the technique of DNA heteroduplex mapping to show mosaicism between bacterial genomes (Simon *et al.*, 1971, Juhala *et al.*, 2000). These studies showed the prevalence of genes that appeared to have associations with prophage inclusion into the bacterial genome. Moron genes were described in the genomes of two *E. coli* strains in 2000 when the genomes were infected with *siphoviridae* HK97 and

HK022 (Juhala *et al*., 2000). Juhala *et al*. (2000) showed that the accessory genome was altered between *E. coli* strains harbouring λ compared to the strains with the *siphoviridae* integrated.

In *E. coli,* these moron genes are often seen in the late gene operons of the phage genome and are believed to aid fitness of the phage (Hendrix *et al*., 2000). An example of a moron gene in a Gram negative bacterium is *SopE* (encoded for by ϕSopE). SopE is a SPI1-dependant translocated protein which is known to support cellular growth and the entry of *Salmonella typhimurium* into mammalian host cells. It is a phage encoded effector which encodes for a type III secretion system in *Salmonella typhimurium* and this system is important for the bacterium during the infection process within the gut (Mirold *et al*., 1999, Hueck, 1998, Galán and Curtiss, 1989, Watson *et al*., 1998).

Table 3.1: Bacteriophage encoded moron genes which may have beneficial advantages for their lysogenic bacterial host cells. Prophage encoded moron genes are believed to have an influence on the bacterial host's survival, as well as promoting for future selection of prophage infected bacterial isolates and therefore, promoting bacterial and phage survival within a particular environmental niche.

Bacteria	Phage	Moron gene of interest	Function	Disease	Reference
Vibrio cholerae	ϕCTX	*CTX* toxin	Alongside toxin production, this phage also encodes for a pathogenicity island. The island encodes a toxin co-regulated pilus which is also a receptor for the CTX phage	Cholera	(Karaolis *et al.*, 1999, Waldor and Mekalanos, 1996)
Corynebacterium diphtheriae	ϕβ	Diphtheria toxin *(DIP0222)*	Produces an exotoxin protein which inhibits protein synthesis by altering elongation factor 2	Diphtheria	(Cerdeño‐Tárraga *et al.*, 2003, Holmes, 2000, Leong and Murphy, 1985)

S. aureus	ϕETA	Exfoliative toxin A	Induces skin exfoliation by splitting the epidermis near the adrenal gland, more common in babies and young children	Scalded skin syndrome	(Ladhani *et al.*, 1999, Dajani, 1972, Melish and Glasgow, 1970, Parker *et al.*, 1955, Lyell, 1956)
Clostridium botulinum	Ceβ	*C1* and *D*	Neurotoxin that affects the nervous system and blocks the passage of a nerve impulse at nerve endings. Leads to paralysis in cranial nerve functions	Botulism	(Eklund *et al.*, 1971, Eklund *et al.*, 1972, Sugiyama, 1980)
E. coli	ϕT4	*52* *58*	Alters host DNA methylation mechanisms which affects the incorporation of foreign phage DNA into the bacterial chromosome	Prevents superinfection of the *E. coli* host with other phages	(Raleigh and Wilson, 1986, Hattman *et al.*, 1985, Fujimoto *et al.*, 1965, Gold *et al.*, 1964, Hausmann and Gold, 1966)

S. aureus	ϕN315	*Staphylokinase* encoded for by *Sak*	Enhances immune resistance as the bacterium activates the Plasminogen/Plasmin system. Which allows the bacteria to avoid host defence systems	Increases *S. aureus* infection rates	(Molkanen *et al.*, 2002, Bokarewa *et al.*, 2006, Wagner and Waldor, 2002, Novick, 2000, Sako and Tsuchida, 1983)
Salmonella typhimurium	Gifsy – 2 Gifsy - 1 Fels – 1	Superoxide dismutases SodC1 SodC111 *nanH*	Sod enzymes catalyse the conversion of superoxide to hydrogen peroxide and provide defence for the bacterial cells against macrophages	Gastroenteritis	(Figueroa-Bossi *et al.*, 2001, Figueroa-Bossi and Bossi, 1999, Figueroa-Bossi *et al.*, 1997, De Groote *et al.*, 1997, Farrant *et al.*, 1997, Fang *et al.*, 1999)
Streptococcus mitis	SM1	*pblA* and *pblB*	Helps bind bacteria to platelets, pblA and plbB are surface encoded	Endocarditis	(Bensing *et al.*, 2001, Sullam, 1994)

			to encourage platelet binding		
Salmonella choleraesuis	ϕ14	Lysogens	Alters the O antigen chain by increasing the length compared to non-lysogenic strains. Leads to increased resistance and virulence	Food poisoing often originating from infected swine ingestion	(Nnalue *et al.*, 1990, Nnalue and Stocker, 1986)
P. aeruginosa	D3	*Wzy*β/A	Confers O-Antigen switching to its bacterial host	Avoid detection by the host's immune system	(Kropinski, 2000, Holloway *et al.*, 1960, Kaluzny *et al.*, 2007)

3.1.2. Phage-bacterial host interactions

3.1.2.1. Bacteriophage and bacterial evolution

Co-evolution between bacteriophages and their bacterial hosts has been proposed within the scientific community for over 30 years. It was suggested that co-evolution allows for the dynamic evolution of two different biological populations in order to inhabit the same environmental niche (Lenski and Levin, 1985, Gómez and Buckling, 2011, Weitz *et al.*, 2005, Rosvall *et al.*, 2006). One of the first mentions of coevolution was in 1983, when it was stated that co-evolution between a bacteria and a parasite depended on the transmission of the parasite (May and Anderson, 1983). It has been proposed that antagonistic co-evolution often occurs between hosts and parasites, this has been observed in both nature and in lab settings (Buckling and Rainey, 2002). These evolution events are believed to play pivotal roles in population dynamics (Buckling and Rainey, 2002, Thompson, 1998) and parasite virulence (Buckling and Rainey, 2002, Thompson, 1998, Bull, 1994).

Buckling and Rainey (2002) demonstrated that a phage of *Pseudomonas fluorescens,* SBW25, was able to co-evolve with its bacterial host for > 300 bacterial generations and proposed that a bacterial-phage arms race may be occurring. This study showed that time-lagged coevolution was occurring between the phage and the bacterial host, as the later generations of the phage communities showed an increase in infectivity profiles and the later generations of bacteria showed an elevated resistance to phage infection. These bacteria-phage arm races have been shown to have an impact on a wide range of environmental processes including global nutrient cycling within the ocean (Suttle, 2007, Fuhrman, 1999, Brüssow *et al.*, 2004), virulence of human

pathogens (Stern and Sorek, 2011, Brüssow *et al.*, 2004), the global climate and the evolution of the biosphere (Comeau and Krisch, 2005, Brüssow *et al.*, 2004).

Antagonistic coevolution can also be referred to as the red queen hypothesis, which was proposed by Van Valen and colleagues in 1973 (Stern and Sorek, 2011, Van Valen, 1973, Lenski and Levin, 1985, Schaffer and Rosenzweig, 1978, Dawkins and Krebs, 1979). The red queen hypothesis proposes that in tight co-evolutionary relationships a change in one community may lead to the near extinction of the other community. This then causes the other community in the relationship to increase its fitness in order to remain a viable entity (Van Valen, 1973, Stern and Sorek, 2011). The specific ways which bacteria and phage can evolve to allow for the maintenance of both communities within a particular evolutionary niche are detailed in section 1.6.5. These include restriction modification, CRISPR/Cas, toxin-antitoxin and abortive phage infection systems.

3.1.3. Polylysogeny

Through the development and implementation of DNA sequencing platforms and continuingly improving sequencing chemistries, pathogenic bacteria have been shown to harbour multiple, inducible temperate bacteriophages in their genetic backbone. These bacterial strains can be classed as polylysogens (Burns *et al.*, 2015, Schuch and Fischetti, 2009, Winstanley *et al.*, 2009, Wang *et al.*, 2010). LES has been shown to harbour multiple novel prophage regions carrying genes with unknown function, figure 3.1. LES harbours 5 inducible prophage regions and 1 defective prophage region which is in contrast to the lab strain PAO1 which harbours only 2 prophage regions (Stover *et al.*, 2000). Winstanley *et al* (2009) showed that disrupting these prophage regions resulted in a reduction of

bacterial fitness in a rat model of chronic lung infection, and so proposed that these prophage regions must offer a beneficial evolutionary advantage for their bacterial host. The pathogenic *E. coli* sakai O157:H7 isolated in Japan has been shown to contain 18 prophages however, not all of these prophages are inducible (Ohnishi *et al.*, 2001). Polylysogeny is not restricted to just Gram negative bacteria as *Streptococcus pneumoniae* has been seen to harbour multiple prophage regions (Ramirez *et al.*, 1999, Obregón *et al.*, 2003).

Burns *et al* (2015) demonstrated that during the initial stages of LES infection in a *Galleria mellonella* insect larvae model, lysogens initially had no beneficial advantage compared to naïve bacterial strains and over time there was in fact an associated 10 % increase in bacterial fitness in the single lysogen relative to the non-lysoge. Importantly, the largest fitness benefit was observed in the double lysogen suggesting that polylysogeny may be adaptive for the bacterial host in terms of growth and antimicrobial resistance (Burns *et al.*, 2015).

In summary, the effects of phages on their bacterial hosts are wide spanning and can have serious consequences on host cell behaviour and disease progression, these are made more apparent in clinical settings. The increased use of antibiotics and alterations in the host's immune response in CF/BR patients also adds a layer of complexity when studying bacterial-phage infections within the lung. These factors make the lung a very stressful environment for both the phage and the bacterial host.

In order for phages to survive in the chronic lung, they need to stay associated with and constantly evolve alongside their *P. aeruginosa* host cells. Their *P. aeruginosa* hosts are also evolving in order to remain within the chronic lung which generates the aforementioned, co-evolution or red queen hypothesis. The complex interaction between phages and their hosts can dramatically alter the

way in which communities react to an environment. It is believed that these bacteria-phage evolution events may influence the patient-patient transmissions of *P. aeruginosa* that are often seen in CF patients.

Due to the severity of bacterial infections crossing into parents or carers of CF patients, the desire to understand the 'antagonistic coevolution' between phages and their bacterial host cells is becoming more pressing. It also poses the question "are temperate bacteriophages a new clinical challenge that need to be considered in order to effectively and efficiently treat chronic respiratory disease patients colonised with *P. aeruginosa*?"

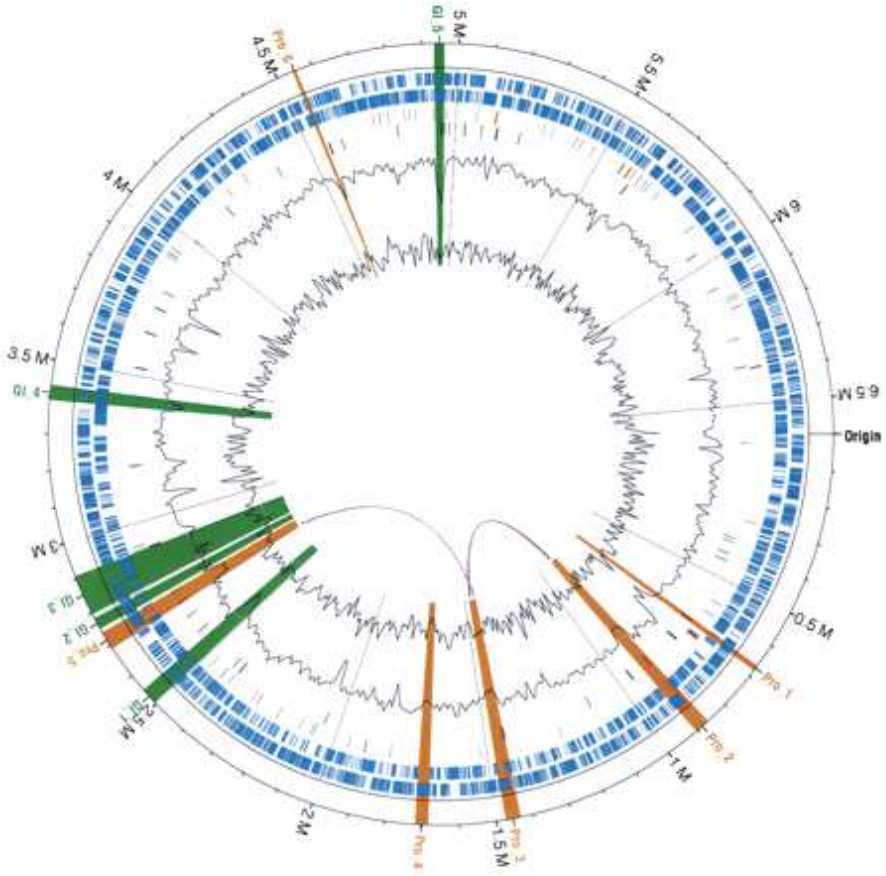


Figure 3.1: Circular chromosomal map of the LES genome (Winstanley *et al.*, 2009). The origin of replication is indicated on the image along with the potential prophage regions (identified in orange) and genomic islands (identified in green). The two most genetically similar prophage regions are marked by the linking purple loops which show the genetic relatedness of the prophage regions. The outer concentric circle represents the GC content of the bacterium whilst the inner circle represents the GC skew. Work has shown that disrupting these prophage regions within the LES genome leads to a reduction in bacterial fitness in a rat model.

3.2. AIMS

The aim of this chapter was to characterise the interaction of possible mixed phage communities induced from described clinical *P. aeruginosa* isolates [CF (n = 47) and BR (n = 47)]. *P. aeruginosa* is referred to as Pa in figures in order to make them and the legends clearer for the reader.

The 47 BR *P. aeruginosa* isolates have been subdivided according to the length of time since diagnosis with BR [under 10 years (< 10, n = 17) and over 10 years (> 10, n = 30)]. The 47 CF samples have been subdivided according to patient age [paediatric CF patients (under 15 years old, n = 10) and adult CF patients (over 15 years old, n = 37)].

The inducible reservoir of phages from each clinical *P. aeruginosa* isolate is focused upon in this chapter. Once these putative mixed temperate phage preparations have been chemically induced away from their bacterial chromosome, it is possible to map the effects of these phages in terms of phage infectivity and bacterial sensitivity towards phage infection. This investigation also identified *P. aeruginosa* phages that were capable of re-infecting their originating *P. aeruginosa* bacterial isolate.

It is hypothesised that the adult CF *P. aeruginosa* isolates and the associated phages will be the most adapted for survival within the chronic lung due to the fact that CF is a lifelong disease. However, it is pertinent to mention that even though CF is a lifelong disorder this doesn't make *P. aeruginosa* colonisation also a lifelong event. An adult CF patient for example, may have only recently been colonised with *P. aeruginosa,* so care needs to be taken when drawing conclusions regarding *P. aeruginosa* colonisation in CF patients.

It is also hypothesised that the phages and *P. aeruginosa* isolates originating from paediatric CF patients and < 10 BR patients will be the least evolved to

survive in this environment due to both these diseases being 'new' diseases. Even though BR occurs later in life it is possible to class it as a 'new' lung disease due to the late onset of clinical symptoms. Again a caveat needs mentioning, just because these diseases are 'new,' this doesn't necessarily mean that *P. aeruginosa* colonisation is also a new event.

3.3. RESULTS

3.3.1. Phage induction and cross infection

Before a cross infection study could be initiated phages were induced from their *P. aeruginosa* host. This was achieved using norfloxacin (NFLX) inductions (section 2.3.3). The induced phage lysates were then utilised in spot assays (section 2.3.3.1.) in order to elucidate the host range of the lysates and to identify any differences in the phage infectivity profiles. It was observed that all the *P. aeruginosa* isolates contained at least one inducible bacteriophage and were capable of infecting more than one *P. aeruginosa* isolate in the cohort. This is one of the first studies to utilise such a large panel of clinical *P. aeruginosa* isolates and focus on the mixed phage populations for each isolate.

When analysing the data presented in this study, it is important to remember the aforementioned caveat – even though CF is a lifelong disease this doesn't predispose the patient to lifetime colonisation with *P. aeruginosa.* This statement is also true when considering the BR results, even though a patient may have only recently been diagnosed with BR there is no clinical data to support the notion that this would be when *P. aeruginosa* would be first isolated from a patient.

The cross infection potential of the mixed phage lysates against the 94 clinical *P. aeruginosa* isolates is shown in figure 3.2. This figure describes the raw data generated during this investigation and it shows that that the CF phages have the broadest host range across the entire *P. aeruginosa* cohort. The BR phages meanwhile have a limited host range. The phages originating from paediatric CF and < 10 BR patients were the least infective and this is perceived to be related to their naivety in the chronic lung environment.

Principle Least Squares Discrimination Analysis (PLS-DA) to look for trends present within the data set whilst using a semi-supervised model

It is difficult to glean information from the cross infection raw data detailed in figure 3.2 and therefore, other approaches are needed to show potential differences in the data. Principle least squares discriminant analysis plots were used, as this modelling approach maximises the variation in the data. This may make it easier to identify differences between infection profiles or disease aetiologies. Bacterial sensitivity towards phage infection was also studied.

Figure 3.3, panel 1 illustrates the differences seen using PLS-DA plots of the infectivity profiles for the different CF aetiologies. These plots are semi-supervised which means that the plots are informed where to look for variation. The data is robust because of the high R^2Y value of 0.72, values > 0.5 are believed to be representative of robust model. Figure 3.3, panel 1 shows that the adult CF phages (blue) align in a discrete group away from the paediatric CF phages (yellow) and this variation is proposed due to the separation along the x-axis.

Figure 3.3, panel 2 describes the infectivity profile differences between the CF and BR phages. In this instance the BR phages (blue) and the CF phages (red)

show clear variation according to their separation along the x-axis. However, there are some CF isolates that align with the BR phages, so indicating that these phages must have similar infection profiles. Three of these four phage lysates originate from paediatric CF *P. aeruginosa* isolates, potentially showing the naivety of these phages (ϕCF214, ϕCF165 and ϕCF213). Unusually, the fourth phage lysate which aligns with the BR lysates originates from a 33 year adult CF patient (ϕCF77). The data presented in this model is again robust because the R^2Y value is 0.77.

As mentioned previously, it was decided to model the sensitivity of the *P. aeruginosa* isolates towards phage infection and to show the maximum variation present within the data via PLS-DA plots. Figure 3.3, panel 3 describes the sensitivity of the CF *P. aeruginosa* isolates to phage infection, the paediatric CF *P. aeruginosa* isolates are represented in red and the adult CF *P. aeruginosa* isolates are represented in blue. There is clear variation between the two groups but there is one exception to this variation because one *P. aeruginosa* isolate originating from a paediatric CF patient aligns with the adult CF *P. aeruginosa* isolates. This shows that these isolates must exhibit similar sensitivity profiles. This paediatric CF *P. aeruginosa* isolate is CF208 and it originates from a 15 year old patient. 15 years is the upper age limit for the paediatric cohort so this leads to the assumption that this bacterial isolate may behave more like adult CF *P. aeruginosa* isolates due to colonisation time within the CF lung. The R^2Y value for this model is 0.66, this indicates that the model is robust as it passes the > 0.5 threshold but the strength of the model is reduced in comparison to panel 1 and 2.

Figure 3.3, panel 4 describes the variation in the sensitivity differences between the CF/BR *P. aeruginosa* isolates; BR isolates are blue and CF isolates are green. The R^2Y value is 0.83. Clear stratification is observed within the model but

two CF *P. aeruginosa* isolates are seen to align with the BR *P. aeruginosa* isolates. These CF *P. aeruginosa* isolates originate from adult CF phages, so this may indicate that this variation from the trend is not driven by naivety. The two CF *P. aeruginosa* isolates are CF136 and CF177 and the patients that these isolates originate from are 30 and 38 years old, respectively. It is interesting to note that these isolates are both epidemic strains of *P. aeruginosa*; CF136 is a *P. aeruginosa* isolate from the Manchester epidemic strain whilst CF177 is a *P. aeruginosa* isolate from the Midlands-1 epidemic strain.

In order to further glean information from the cross-infection raw data, PLS-DA models were generated to look for variation between the mucoid and non-mucoid *P. aeruginosa* isolates with phage infectivity and bacterial sensitivity again being modelled in order to show the maximum variation present, shown in figure 3.4. In all the PLS-DA models, the mucoid isolates are shown in red and the non-mucoid isolates are shown in blue.

Figure 3.4, panel 1 describes the phage infectivity differences when the BR phages are stratified according to their host's phenotype. The isolates do not form discrete groups on the PLS-DA which may indicate that there is less variation present between the BR phages infectivity and the mucoid phenotype is not a clear driving factor. The R^2Y value is 0.67, which shows that the model is robust as it is above the > 0.5 threshold.

Figure 3.4, panel 2 details the variation in the infectivity profiles of the CF phages when they are stratified according to the presence/absence of a mucoid phenotype in their host, the R^2Y value is 0.72. There are two outliers in this PLS-DA plot which fall outside of the 95 % confidence eclipse, both these outliers are phages induced from mucoid *P. aeruginosa* isolates. ϕCF54 originates from an adult CF patient (17 years) whilst ϕCF187 originates from a paediatric CF patient

(2 years). This suggests that age differences are not driving the differences in the phage infectivity profiles.

Figure 3.4, panel 3 describes BR *P. aeruginosa* isolates sensitivity towards phage infection when the BR isolates exhibit either a mucoid or a non-mucoid phenotype. In a similar trend to the observation in figure 3.4, panel 1 there appears to be less stratification between the two groups. This would indicate that the variation in the data is not due to the bacterial isolates phenotype. The R^2Y value for this PLS-DA plot is 0.67.

The final panel in figure 3.4 determines the variation in the bacterial sensitivity profiles towards phage infection when the CF *P. aeruginosa* isolates are grouped according to the presence/absence of a mucoid phenotype. This plot indicates that a mucoid *P. aeruginosa* isolate aligns with the non-mucoid *P. aeruginosa* isolates. This mucoid *P. aeruginosa* isolate is CF208 and it originates from a paediatric CF isolate which potentially indicates that time is a discriminating factor in the variation seen in this plot. The R^2Y value is 0.73.

Statistical modelling of the data to support the data previously presented via PLS-DA plots

Figure 3.5 describes the cross infection data using ANOVA analysis. This method uses nestedness as a measure of network order where a highly nested pattern describes a hierarchy from generalists (broad host range) to specialists (narrow host range) for both species. The binmatnest value is the deviation of the network pattern from an optimal nested pattern of the same dimensions; values are on a scale from 0 (highly nested) to 100 (not nested at all). These plots were generated in collaboration with Professor Mike Brockhurst at the University of York, York, UK.

Panel 1 represents that ability of the BR/CF phages to infect the entire *P. aeruginosa* cohort. It is possible to propose from these data that the adult CF phages are the most infective across the *P. aeruginosa* cohort. This was illustrated by the higher gradient of infections in the nested plot for the CF phages against both bacterial backgrounds (an infection is indicated via a black mark).

When the data was modelled using the mucoid phenotype (panel 2), then it was observed that only the mucoid BR *P. aeruginosa* isolates (n = 22) generated any significant data. The findings from these nested plots showed that the BR phages could infect mucoid BR *P. aeruginosa* isolates at a higher rate compared to non-mucoid BR *P. aeruginosa* isolates. This may be indicative of the environment in the lower lung of BR patients and so the phages will have evolved in order to infect and survive these adverse conditions.

Panel 3 shows the associated statistical information relating to these plots. The use of ANOVA to describe these data was important for this analysis due to its ability to confer statistical weight to the results and findings proposed.

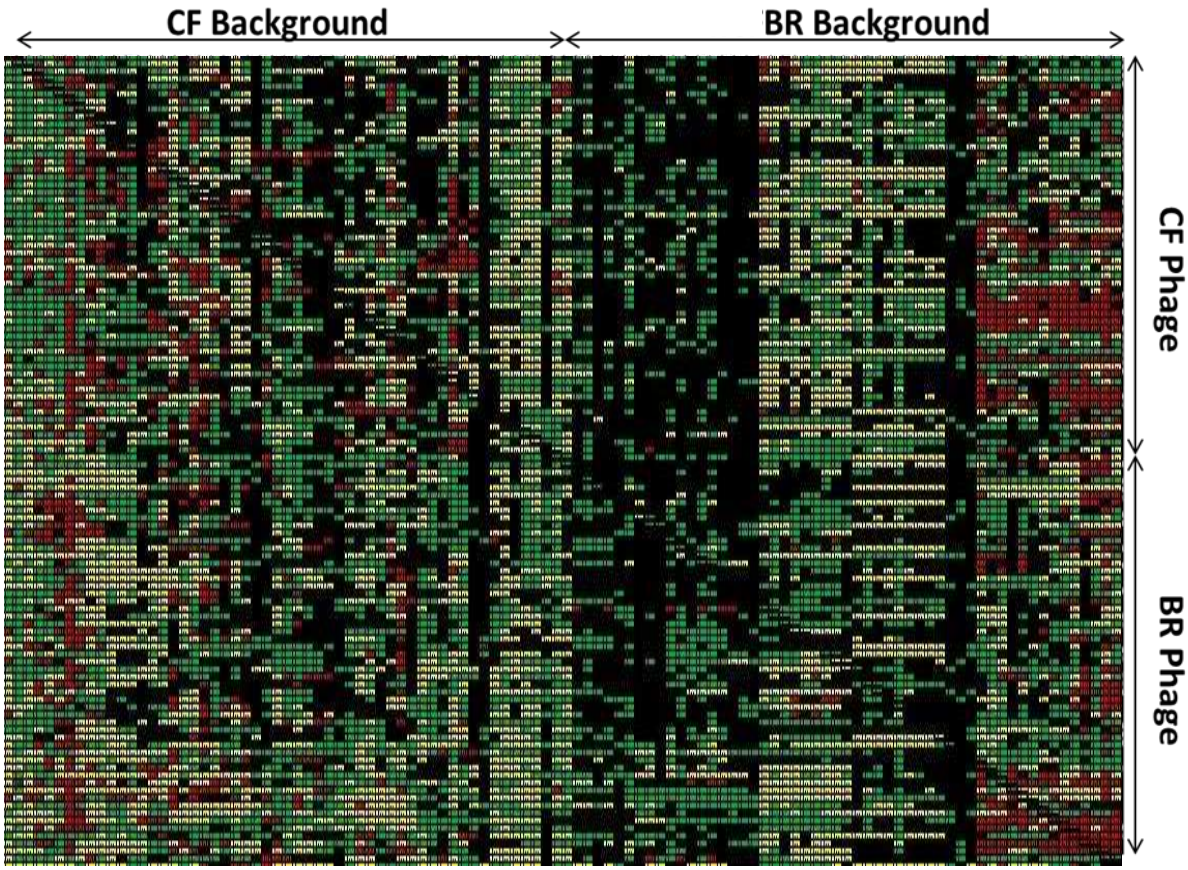


Figure 3.2: Raw cross infection data obtained showing the > 3000 reactions involved in generating this data. Excel derived table showing cross infection data. Black cells illustrate phage lysates that were incapable of infection on that particular bacterial host. Green cells represent infection and infection assays showing total lysis of the bacterial host. Yellow cells represent infection including a central, cleared lysed zone and a turbid halo on the bacterial host. Red cells represent turbid zones of clearing.

CF background refers to *P. aeruginosa* isolates originating from CF patients whilst BR background refers to *P. aeruginosa* isolates originating from BR patients.

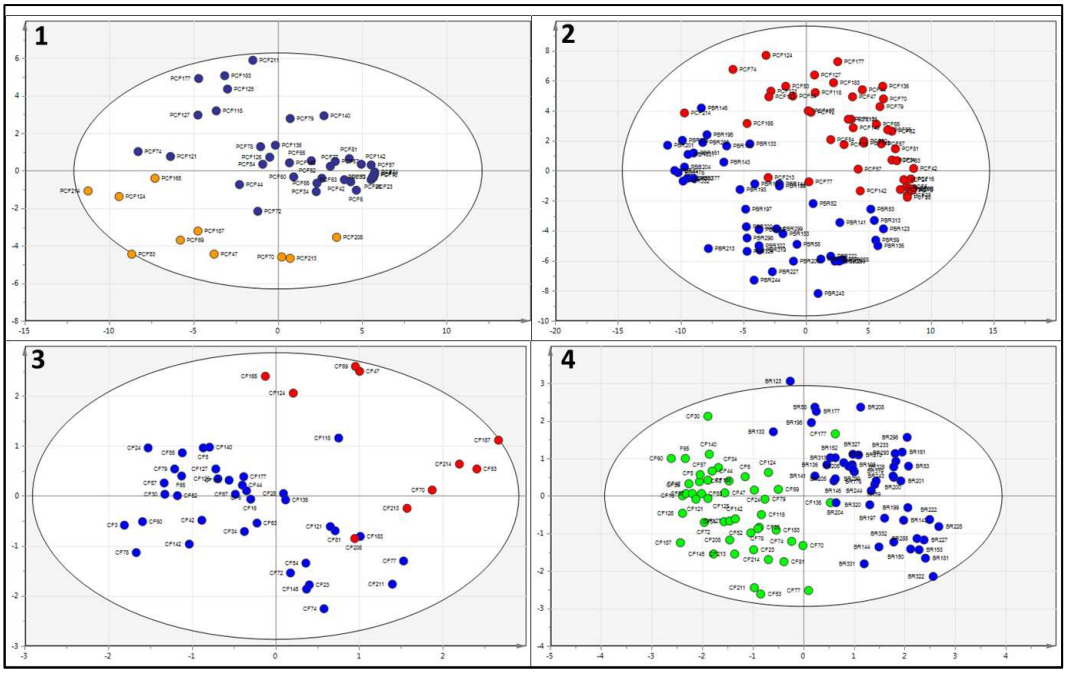


Figure 3.3: PLS-DA plots to show the differences in phage infectivity profiles and bacterial sensitivity profiles between the CF/BR isolates and upon stratification of the CF isolates according to age. Panel 1 shows the differences in the phage infectivity profiles between the paediatric CF phages (yellow) and the adult CF phages (blue) - R^2Y is 0.72. Panel 2 shows the total differences in phage infectivity for the BR phages (blue) and the CF phages (red) - R^2Y is 0.77. Panel 3 shows the bacterial sensitivity profiles comparing the paediatric CF phages (red) and the adult CF phages (blue) - R^2Y value is 0.66. Panel 4 shows the bacterial sensitivity profiles comparing the CF (green) and BR phages (blue) - R^2Y value is 0.83.

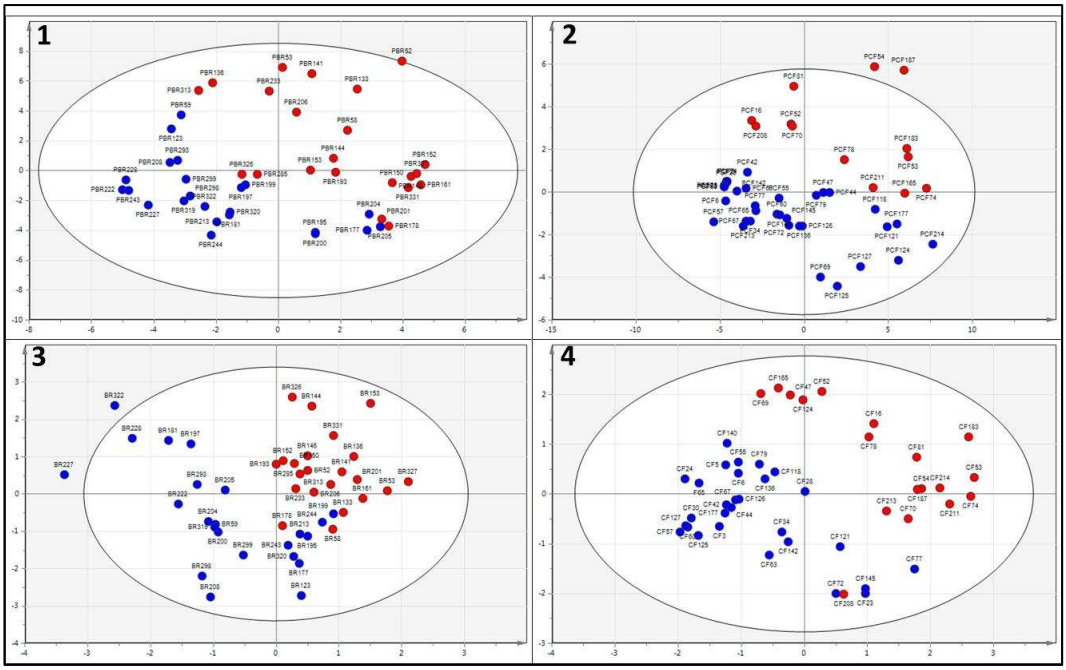
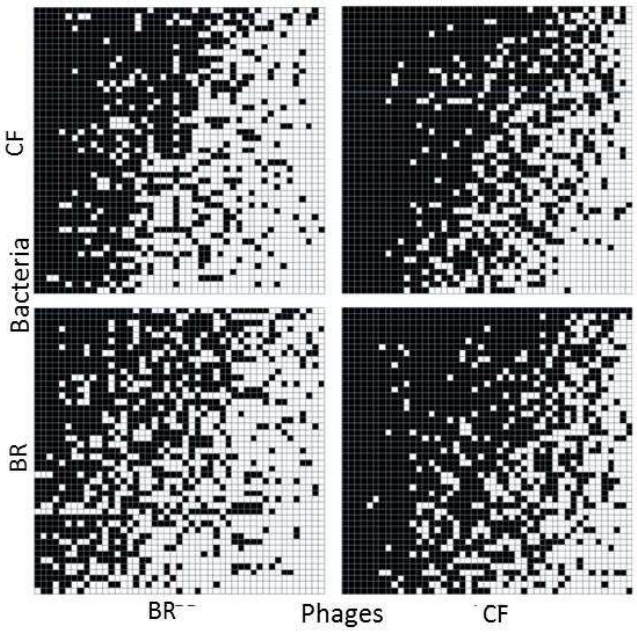


Figure 3.4: PLS-DA plots to show differences in phage infectivity and bacterial sensitivity when the isolates are stratified according the presence/absence of a mucoid phenotype. All the mucoid isolates are shown in red and the non-mucoid are shown in blue. Panel 1 describes the variation in the phage infectivity profiles of the BR phages, R^2Y value is 0.67. Panel 2 describes the differences seen between the phage infectivity profiles for the CF phages upon stratification for a mucoid phenotype, R^2Y value is 0.72. Panel 3 describes the variation in the BR isolates sensitivity to phage infection, R^2Y value is 0.67. Panel 4 shows the difference observed in the sensitivity profiles for the CF isolates, R^2Y value is 0.73.

1



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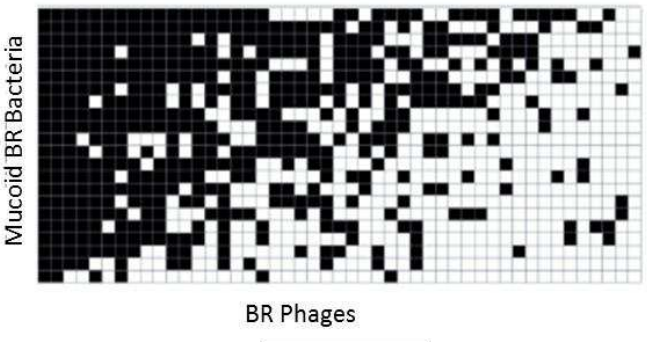


3

	Connectance	Nestedness (binmatnest)
(1) Φ CF V CF	0.659	26.9
(1) Φ BR V CF	0.528	28.2
(1) Φ BR V BR	0.486	38.5
(1) Φ CF V BR	0.693	24.9
(2) All Φ BR x Mucoid BR	0.502	28.6
All Φ BR x Non Mucoid BR	0.494	39.9

Figure 3.5: Nestedness and Connectance plots to map the cross infection data from the mixed temperate phage communities induced from the 94 clinical *P. aeruginosa* isolates. Analysis of the data has been undertaken by Rosanna Wright and Michael Brockhurst (York University, UK). Each black square represents an infection event between a phage lysate (x-axis) and a *P. aeruginosa* isolate (y-axis). A white square indicates when no infection occurred. Panel 1 shows the binary nested network for the 94 mixed phage lysates compared against the 94 *P. aeruginosa* isolates, the quadrants are of equal size. Panel 2 shows the binary nested network of the mucoid BR *P. aeruginosa* isolates (n = 22) against the entire cohort of induced mixed temperate phage lysates. The mucoid data was derived from the associated clinical data for each of the *P. aeruginosa* isolates involved in this investigation. Panel 3 describes the metric values for the results shown in panels 1 and 2.

3.3.2. Isogenic phage infection of originating bacterial isolates

When the cross infection data was modelled, it was determined that certain phages were capable of infecting their originating host. It has also been seen that the *E. coli* phage 24_B can infect its originating *E. coli* host cell due to the presence of a novel integrase (Fogg *et al.*, 2007)

Figure 3.6 illustrates that 66 % of CF phage lysates cause an infection on their originating host bacteria whilst 49 % of BR phage lysates cause an infection on their originating host. Panel 1 shows the stratification of the phage lysates that can re-infect their originating *P. aeruginosa* lysate when looking only at CF and BR. Panel 2 further subdivides these data by age/time to look for differences in the phages that have the ability to re-infect their originating *P. aeruginosa* host. 84 % of the adult CF phage lysates could re-infect their originating *P. aeruginosa* host whilst only 16 % of paediatric CF phage lysates had this ability. When looking at the stratification of the BR phage lysates it was seen that 30 % of the < 10 BR phage lysates could infect their originating host compared to 70 % of the lysates > 10 BR could infect their originating *P. aeruginosa* host. These results may show a link between the ability to re-infect their originating bacterium as being an adaptive or evolved trait that is linked to disease progression.

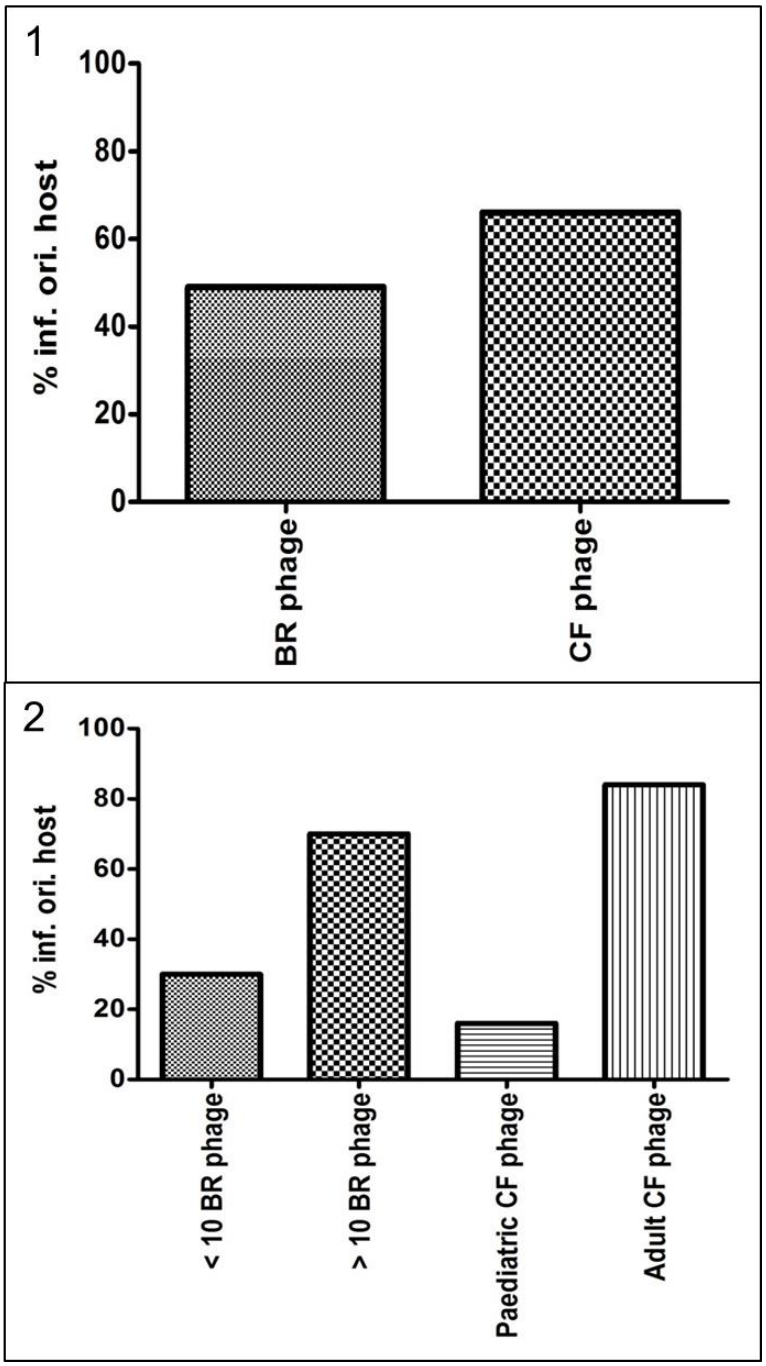


Figure 3.6: Ability of phages in this investigation to re-infect their originating host cell. Graphical representation of the percentage of phages that are capable of re-infecting their originating host (% inf. ori. host = % infection of originating host); this image is taken from (Tariq *et al.*, 2015). Panel 1 shows the percentage of phage lysates from BR and CF *P. aeruginosa* isolates. Panel 2 shows further stratification of the data shown in panel A according to the clinical data.

3.3.3. Stratification of the cross infection results based on the clinical data – phage infectivity

The ability of each phage lysate sub-group to infect *P. aeruginosa* hosts from the various aetiologies has been illustrated in figure 3.7. The mean rates of infectivity and the host range distribution of the phage infections are detailed in table 3.2. The phages have been characterised according to the clinical data available, so it has been possible to elucidate any potential differences in the infection capabilities of the phage lysates according to patient age (CF) and length of time since diagnosis (BR). The previously mentioned caveat is also applicable in the analysis of this data set. The data is also further subdivided so that it is possible to study differences in the infectivity of a particular clinical phage group against the various subgroups of the *P.aeruginosa* isolates.

These data indicates that the adult CF phages have the greatest mean rate of infectivity across the entire clinical *P. aeruginosa* cohort, possibly due to these phages being long term colonisers of the chronic lung environment. The 'newer' colonisers, paediatric CF phages and < 10 BR phages, showed a lower rate of infectivity. It is proposed that time influences the infectivity patterns seen, as the longer that the phage has had to colonise an environment correlates to a greater infectivity range being observed.

Table 3.2: Infectivity rates for each phage lysate against the various bacterial aetiologies. Mean rates of phage infectivity and the host range distribution for each aetiological subgroup of phages against the different *P. aeruginosa* hosts involved in this thesis.

For each phage infection against a particular *P. aeruginosa* background, a % score was given in order to elucidate the host range of the particular phage lysate. From these values, the mean % of infection could be calculated as could the distribution of infection around the mean.

Bacterial host	Phage lysate	Panel	Mean rate of infectivity (%)	Distribution of infectivity
Paediatric CF	Paediatric CF	1	11	9
	Adult CF		68	38
	< 10 BR		57	37
	> 10 BR		49	49
Adult CF	Paediatric CF	2	36	36
	Adult CF		55	44
	< 10 BR		63	52
	> 10 BR		41	41
< 10 BR	Paediatric CF	3	49	34
	Adult CF		78	63
	< 10 BR		63	51
	> 10 BR		54	54
> 10 BR	Paediatric CF	4	48	34
	Adult CF		76	49
	< 10 BR		56	42
	> 10 BR		45	38

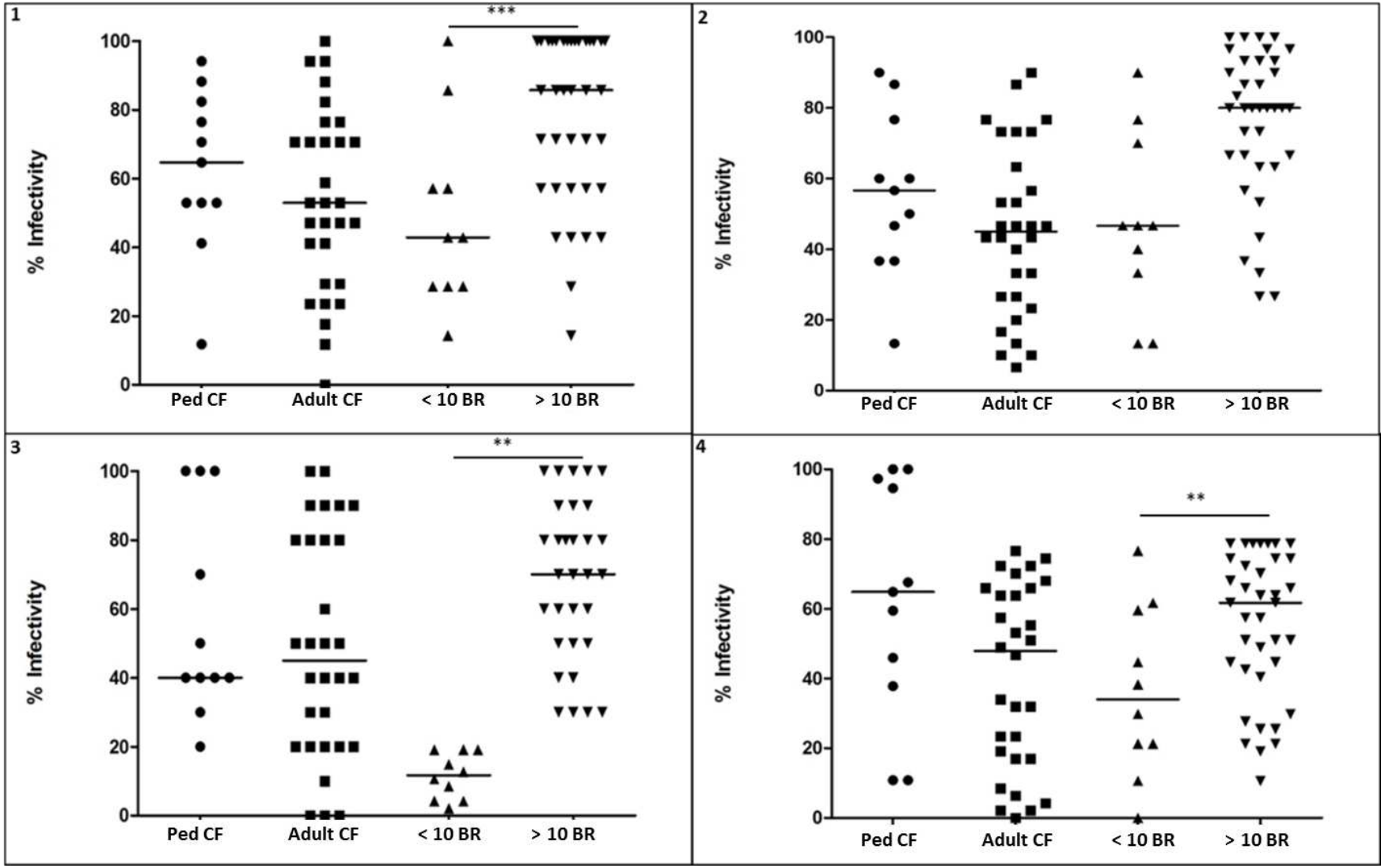

1
***
% Infectivity
100
80
60
40
20
0
Ped CF
Adult CF
< 10 BR
> 10 BR
2
% Infectivity
100
80
60
40
20
0
Ped CF
Adult CF
< 10 BR
> 10 BR
3
**
% Infectivity
100
80
60
40
20
0
Ped CF
Adult CF
< 10 BR
> 10 BR
4
**
% Infectivity
100
80
60
40
20
0
Ped CF
Adult CF
< 10 BR
> 10 BR

Figure 3.7: Infection capabilities of each phage aetiological group against the various bacterial aetiological groups. Non parametric t tests were utilised with two tailed p values shown in order to display the most discrete statistical significant data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001.

Panel 1 shows the challenge of paediatric CF phage lysates onto the complete *P. aeruginosa* panel.

Panel 2 shows the addition of adult CF phage lysates onto the complete *P. aeruginosa* panel.

Panel 3 shows the addition of < 10 BR phage lysates onto the complete *P. aeruginosa* panel.

Panel 4 shows the addition of > 10 BR phage lysates onto the complete *P. aeruginosa* panel.

3.3.4. Sensitivity to phage infection by the *P. aeruginosa* panel

The sensitivity of each of the *P. aeruginosa* isolates to be infected by the NFLX induced phage lysates is important alongside phage infectivity, as it shows the permissiveness of the bacteria to phage infection from the varying evolving disease states. These data are shown in figure 3.8. The mean rate of sensitivity for each *P. aeruginosa* isolate to phage infection is detailed in table 3.3 and the host range distribution of sensitivity is also described. Care needs to be taken when analysing this data as these subgroups of clinical data may not relate to length of time of *P. aeruginosa* colonisation of the CF/BR lung.

When looking at the sensitivity of the *P. aeruginosa* hosts to phage infection on figure 3.8, it is interesting to note that all the sensitivity profiles are similar for the specific *P. aeruginosa* backgrounds, regardless of the clinical origin of the phage lysate. The adult CF *P. aeruginosa* isolates are not only the most resistant to phage infection but they have the smallest difference relating to sensitivity. It is also important to note that sensitivity to phage infection decreases over time in the CF population.

Table 3.3: Sensitivity of the *P. aeruginosa* isolates to phage infection. The mean rate of sensitivity and the distribution of the sensitivity of *P. aeruginosa* isolates towards phage infection, subdivided according to the associated clinical data.

For each sensitivity profile for a particular *P. aeruginosa* background, a % score was given in order to elucidate how sensitive the *P. aeruginosa* strain was towards phage infection, the phages were subdivided into groups according to their origin. From these values, the mean % of infection could be calculated as could the distribution of infection around the mean.

P. aeruginosa	**Phage lysate**	**Panel**	**Mean rate of sensitivity (%)**	**Distribution of sensitivity**
Paediatric CF	Paediatric CF	1	54	24
	Adult CF		46	36
	< 10 BR		44	34
	> 10 BR		48	38
Adult CF	Paediatric CF	2	58	18
	Adult CF		70	19
	< 10 BR		75	24
	> 10 BR		76	24
< 10 BR	Paediatric CF	3	57	3
	Adult CF		63	26
	< 10 BR		63	53
	> 10 BR		56	38
> 10 BR	Paediatric CF	4	49	22
	Adult CF		52	15
	< 10 BR		54	30
	> 10 BR		45	35

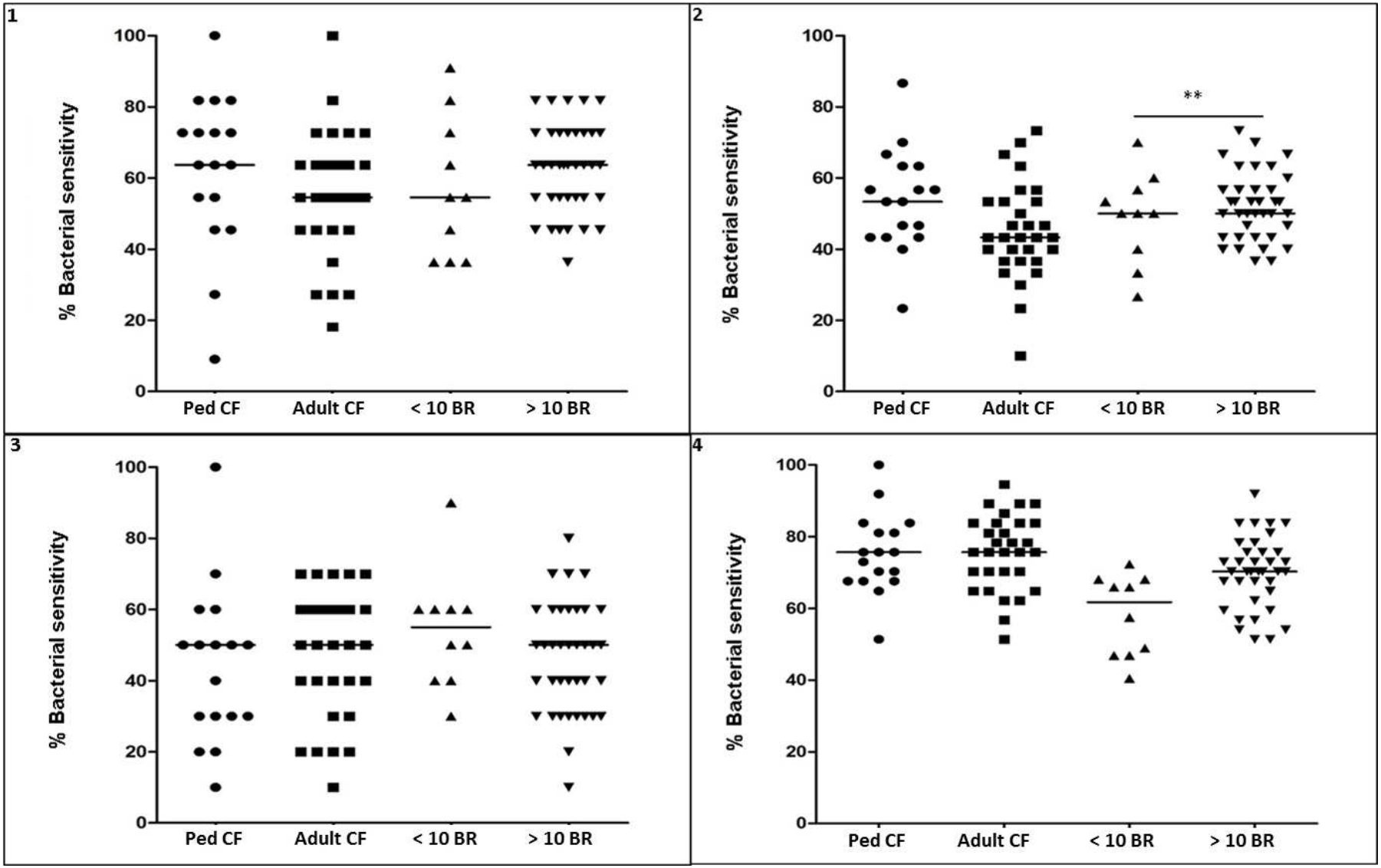

1
% Bacterial sensitivity
100
80
60
40
20
0
Ped CF
Adult CF
< 10 BR
> 10 BR
2
% Bacterial sensitivity
**
Ped CF
Adult CF
< 10 BR
> 10 BR
3
% Bacterial sensitivity
Ped CF
Adult CF
< 10 BR
> 10 BR
4
% Bacterial sensitivity
Ped CF
Adult CF
< 10 BR
> 10 BR

Figure 3.8: Varying sensitivities of each bacterial isolate to phages originating from the various clinical aetiologies. Non parametric t tests were utilised with two tailed p values shown in order to display the most discrete statistical significant data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001. The different origins of the phage lysates are indicated on the x axis of the plots and the bacteria host which the sensitivity tests were undertaken on is represented by the panel number.

Panel 1 shows the addition of phage lysates from the varying aetiologies onto the paediatric CF *P. aeruginosa* isolates.

Panel 2 shows the addition of phage lysates from the varying aetiologies onto the adult CF *P. aeruginosa* isolates.

Panel 3 shows the addition of phage lysates from the varying aetiologies onto the < 10 BR *P. aeruginosa* isolates.

Panel 4 shows the addition of mixed phage lysates from the varying aetiologies onto the > 10 BR *P. aeruginosa* isolates.

3.3.5. Is phage infectivity linked to polylysogeny?

We have demonstrated different infectivity profiles between phages from each stratum of the chronic respiratory disease cohort (section 3.3.1). Due to previous research on LES and its multiple inducible phages (Winstanley *et al.*, 2009), it was presumed that polylysogeny is important in bacterial survival and evolution in the chronic lung. We cannot determine from the cross-infection raw data whether the broad host range of phages isolated from the later stages of disease is linked to a specific phage or is simply linked to a higher incidence of polylysogeny. Single plaque purified five phages from each of the four clinical aetiologies were used to determine whether the results were due to a single predominant phage. Figure 3.9 details the total change in the cross infection profiles for the mixed and single phage infection across the 94 *P. aeruginosa* isolates.

Figure 3.9 shows that mixed phage lysates mostly exhibit an elevated propensity for infection in comparison to the single, plaque purified phages. Upon stratification for the different clinical aetiologies the same trend was seen apart from in the paediatric cohort. In this instance, the plaque purified phages had a greater infection profile. With the exception of the paediatric cohort, the elevation in the infection profiles of the mixed phage lysates would lead to the hypothesis that the *P. aeruginosa* bacterial hosts may harbour multiple inducible phages in their chromosomal DNA. In LES it has seen that harbouring multiple prophages increases the bacterium's overall infectivity, so it may be possible to propose that this is also within our *P. aeruginosa* cohort (Winstanley *et al.*, 2009). This therefore, may explain how *P. aeruginosa* becomes the primary and predominant coloniser of the chronic lung environment. Polylysogeny is a currently overlooked area in the field of chronic lung diseases and it may be the driving factor behind the results described previously in this chapter.

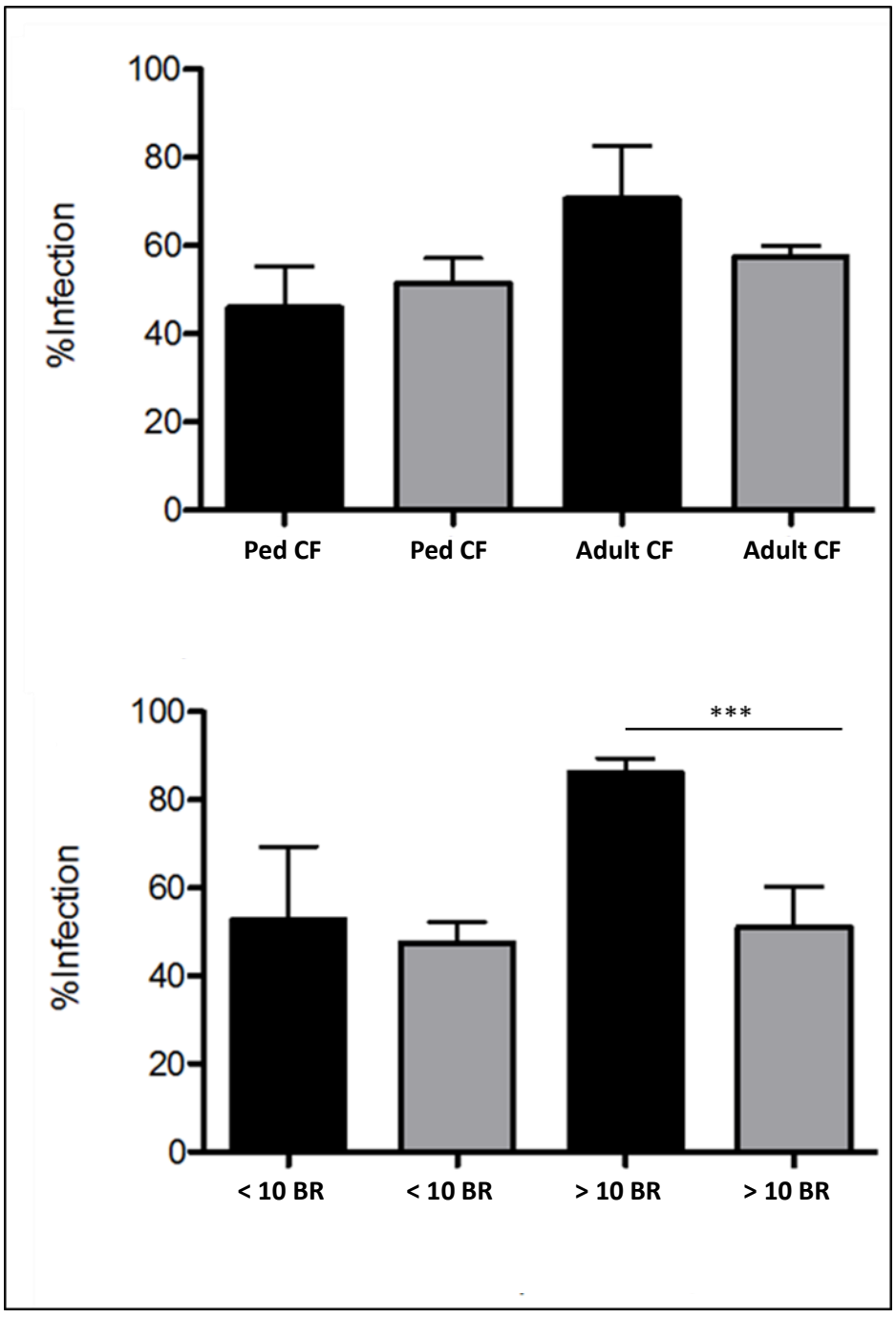

100
80
60
40
20
0
%Infection
Ped CF
Ped CF
Adult CF
Adult CF
100
80
60
40
20
0
%Infection
***
< 10 BR
< 10 BR
> 10 BR
> 10 BR

Figure 3.9: The additive effect of phage infections compared to the effect of singular plaque purified phage infections. Twenty bacteriophages were selected (5 from each of the 4 clinical aetiologies) and were plaque purified. These plaque purified phages were then cross-infected against the 94 clinical *P. aeruginosa* isolates; CF n = 47 and BR n = 47. Black bars indicate when the mixed phage lysate was used whilst the grey bars indicate the use of single, plaque purified phages.

The results from the single phage cross infections were compared to the previous results which utilised mixed phage communities in order to show if the infection patterns were due to additive phage infection or a single and predominant plaque purified phage. Panel 1 illustrates the infection profiles for the CF isolates when comparing mixed and purified phages. Panel 2 illustrates the infection profiles for the BR isolates when comparing mixed and purified phages

3.4. DISCUSSION

These data contribute towards one of the largest reported studies looking at the relationship and interaction between temperate bacterial viruses and their bacterial hosts. This study initially focused on mixed temperate phage communities, as this was believed to represent the most realistic scenario for studying the interactions of the entire viral community with their bacterial hosts *in vitro*. However, in order to study more detailed interactions, single phages were purified and used for further downstream applications. Phages may represent a potential novel area of research when trying to elucidate the progression and evolution of chronic lung diseases. This is due to the ability of phages to enhance the spread of DNA around a community and so enhance the levels of recombination.

This work is original due to its large scale nature and a key finding was that multiple phages were isolated from *P. aeruginosa* isolates originating from the later stages of CF and BR and that these phages were associated with an increased bacterial host range. This could represent the possible impact that polylysogeny has during *P. aeruginosa* infections. These data also support work previously published by Buckling and Rainey (2002), which showed the development of antagonistic co-evolution between bacteria and phage. It is believed that this work shows antagonistic co-evolution as it is assumed that the phage communities evolve and adapt alongside their *P. aeruginosa* host, this adaptation and evolution is perceived to aid the survival and retention of both biological entities in the chronic lung setting.

It was interesting to note that the phages isolated from the later stages of disease, were able to readily infect and replicate in *P. aeruginosa* isolates from the “earlier” disease isolates. It was observed that all the *P. aeruginosa* isolates in this cohort harboured inducible temperate bacteriophages which could infect at

least one of the *P. aeruginosa* isolates. It is possible to hypothesise that this ability to infect the more naïve isolates of *P. aeruginosa* provides an advantage for the evolved phages, as it increases their host range ensuring their longevity within the chronic lung environment. The CF phages were also seen to infect BR *P. aeruginosa* isolates at an elevated level suggesting that the CF phages are more evolved to avoid the host defence systems of BR *P. aeruginosa* isolates. It is also possible to speculate that this evolution is due to the fact that CF is a lifelong disorder and that the phages have acquired efficient anti-CRISPR genes which target a wide range of CRISPR/Cas defence systems. It could also be proposed that the BR *P. aeruginosa* isolates are not as evolved in terms of phage defence systems, as the bacterial isolates are new colonisers of the chronic lung environment and so may not be used to constant bombardment by phages. All this work emphasises the complexity of the lung microbiome and the evolving nature of the chronic lung.

ANOVA models to show nestedness and connectance, generated statistically significant results for the cross-infection raw data (section 3.3.1.). These analyses lead to the proposition that the adult CF phages were the most infective across the two *P. aeruginosa* cohorts. It also showed that the BR phages could infect BR *P. aeruginosa* mucoid isolates (n = 22) at elevated levels compared to non-mucoid isolates and this led to a hypothesis that the BR phages are used to encountering mucoid *P. aeruginosa* isolates within the chronic lung and therefore, have evolved infection strategies to overcome this phenotypic alteration.

When studying PLS-DA plots to look at the variation in the data relating to phage infectivity, it was seen that the adult CF phages formed a discrete group away from the paediatric CF phages. This would indicate that phage infectivity profiles alter as the bacteria evolve within the chronic lung. PLS-DA plots were also used

to show the total difference in the phage infectivity profiles for the CF and BR phages. These two cohorts showed maximum variation upon modelling and this may be an example of evolution of the phages which colonise the chronic lung. Some CF phages were seen to align with the BR phages in terms of their infectivity profiles but 75 % of these phages originated from paediatric CF phages. This indicated to us that naivety was driving the variation observed. Similar variation patterns were seen when the PLS-DA plots were used to show differences in bacterial sensitivity towards phage infection. All the PLS-DA plots shown in this chapter have R^2Y values greater than 0.5 which shows the robustness of the model.

A large percentage of the phages in this investigation do not conform to the typical lambda model of phage infection, as they have the ability to re-infect their originating host cell. The lambda model proposes that upon infection with one phage the bacterium is immune to further phage infection events including self-infection (Kaiser and Jacob, 1957, Ptashne, 1967). The *E. coli* phage 24_B also exhibits this atypical non-lambda infection pattern as it has been demonstrated that this phage is able to infect its host cell multiple times and at high frequencies. Potentially increasing the pathogenicity of its host cell through each passing round of infection due to gene addition (Smith *et al.*, 2012). Phages induced from *P. aeruginosa* isolates originating from adult CF patients had the greatest ability to re-infect their originating host, which supports earlier findings that the CF phages isolated from later disease isolates were the most infective. The proposal that superinfecting phages have a higher propensity for polylysogeny is supported in these data, as the older *P. aeruginosa* isolates harboured on average more than one inducible phage and also had an elevated propensity to re-infect their originating host cell.

It was proposed that the adult CF phages had the greatest mean infection rates due to their long term colonisation of the lower lung. This long term colonisation may have allowed the phages to evolve in order to harbour effective anti-bacterial systems such as alteration of the sequence recognised in the restriction-modification systems. The phages may have also evolved in order to infect the *P. aeruginosa* isolates through the utilisation of a different cellular target to aid phage adsorption.

When looking at bacterial sensitivity it was seen that in the CF isolates this was reduced as time progressed whereas, the sensitivity increased in BR isolates. This would indicate that the BR isolates are evolving in order to overcome phage infectivity by the more evolved and complex phages. This would also indicate that the evolutionary distance between the *P. aeruginosa* isolates maybe increasing in relation to their clinical aetiology. It may also indicate that the isolates have different survival strategies which alter the direction of evolution and adaption of the isolates within the chronic lung. Low sensitivity profiles may indicate that some of the phages in this study may have alternative infection capabilities or targets, which may not be present on all the bacterial hosts which could therefore, prevent observation of an infection.

In order to confirm whether polylysogeny was driving the results seen in this thesis it was decided to undertake the cross infection study utilising single plaque purified phages. It was observed that the mixed phage lysates had an elevated ability to generate an infection. Upon stratification of both the mixed and single phage lysates into clinical aetiological groups, it was observed that the same trend was still apparent even in the smaller cohorts. The only exception to the trend was the paediatric CF single, where the plaque purified had greater infectivity capabilities against the entire *P. aeruginosa* cohort in comparison to the mixed paediatric CF phage lysates. It is possible to hypothesise that

polylysogeny is providing the bacteria with a selective advantage within the chronic lung, this can be proposed because greater infectivity profiles are observed when using mixed lysates compared to single, plaque purified phages.

All these data presented here indicate the effect of phages on *P. aeruginosa* host cells whilst also indicating that prophage incorporation alters the bacterial response to the environment in the lower lung. It also leads to the conclusion that phages may be a new clinical target of interest for clinicians treating CF and BR patients who are chronically colonised with *P. aeruginosa*.

4. PROPHAGE MEDIATED CHANGE IN PHENOTYPE AND ANTIMICROBIAL RESISTANCE

4.1. INTRODUCTION

In section 1.5.4 the presence of multiple inducible prophage regions within the chromosome of *P. aeruginosa* is described. Integration of new prophages can offer positive or negative benefits with respect to bacterial fitness or evolutionary selection but these benefits are dependent on the health and fitness of the bacterial cell, as discussed in section 3.1.2. This chapter focuses on how prophages influence the bacterial host and how this may drive selection and longevity in the lower lung. A key focus is phage encoded antibiotic resistance and phage mediated increases in growth rate. This chapter also focuses on bacterial motility through twitching and how phage infection can mediate microbial movement in the lower lung.

4.1.1. Antibiotic resistance development in *P. aeruginosa*

Antibiotic resistance is becoming a global concern with reported bacterial resistance to all major classes of antibiotics (WHO, 2015). Some well-known examples of multidrug resistant (MDR) Gram positive bacteria include methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* species (VRE) (Hancock and Knowles, 1998, Hancock and Speert, 2000, Enright *et al.*, 2002).

The focus of this thesis is *P. aeruginosa* and table 4.1 is adapted from Hancock *et al* (2000). It shows the antibiotics that are commonly used to treat Pseudomonad infections. It is now common practice in clinics to treat *P. aeruginosa* infections with a combination of drugs from the various classes, most

commonly a β lactam and an aminoglycoside (Hancock and Speert, 2000, Vardakas *et al.*, 2013, Tamma *et al.*, 2012). *P. aeruginosa* sometimes requires a combination of mutations to gain resistance to various classes of antibiotics so multiple drug administration can slow down resistance acquisition (Livermore, 2002). It is believed that the mutations in *P. aeruginosa* accrue in an additive manner which aids the development of the MDR phenotype (Lee *et al.*, 2000, Livermore, 2002). The hypermutator phenotype of *P. aeruginosa* may explain the alterations in resistance profiles that are seen during longitudinal studies. In these studies, the bacterium can convert to an antibiotic resistant phenotype during antimicrobial treatments but revert back to the less costly antibiotic sensitive profile when the external stimuli is removed or reduced (Oliver *et al.*, 2000). *P. aeruginosa* as previously described often has a mucoid phenotype upon isolation from the chronic lung environment. This mucoid phenotype has the potential to reduce the effects of antibiotic treatment as the mucoid exopolysaccharide (EPS) layer may interfere with the penetration of certain antibiotics (Slack and Nichols, 1981, Oliver *et al.*, 2000, Pressler *et al.*, 2006, Hoffmann *et al.*, 2005, Costerton *et al.*, 1999, Ciofu *et al.*, 2001, Worlitzsch *et al.*, 2002).

Variation in the antimicrobial resistance profiles of *P. aeruginosa* within populations colonising the CF lung has been observed (Mowat *et al.*, 2011). It has also been observed that treating *P. aeruginosa* infections with sub-inhibitory concentrations of antibiotics leads to greater diversity within antimicrobial resistance profiles of *P. aeruginosa* isolates (Wright *et al.*, 2013).

S. aureus phages have been seen to transfer phage encoded resistance genes to non–*aureus* staphylococcal strains such as *Staphylococcus epidermidis* and *Staphylococcus xylosus*, they have also been seen to transfer this staphylococcal phage encoded resistance to other bacterial genera such as

Listeria monocytogenes (Quiles-Puchalt *et al.*, 2014, Chen *et al.*, 2015, Chen and Novick, 2009, Kenzaka *et al.*, 2010). Modi *et al* (2013) demonstrated that when mice were treated with antibiotics, their phage metagenomes became enriched for genes involved in antibiotic resistance (Modi *et al.*, 2013). When the faeces of healthy human participants was studied via metagenomics, a large number of genes relating to antibiotic resistant genes were observed in bacteriophage DNA including bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, *mecA, qnrA* and *qnrS* (Quirós *et al.*, 2014). These studies indicate that phages may play a potentially crucial role in the spread of resistance around a population, through acquisition of bacterial chromosomal DNA and the transfer of this material to another susceptible bacterial host.

It is believed that the large genome size, plasticity and the ability to carry large numbers of plasmids is the cause of the antimicrobial resistance in *P. aeruginosa*. *P. aeruginosa* harbours ~5570 genes (strain dependent) with a large proportion of these genes being assigned to regulation. This is consistent with the observation that most of the antibiotic resistant *P. aeruginosa* strains characterised have regulatory gene mutations (Hancock and Speert, 2000). Sherrard *et al* (2014) generated a visual representation of the various antibiotic resistance mechanisms exploited by *P. aeruginosa* and other Gram negative bacterium as shown in figure 4.1. *P. aeruginosa* antibiotic resistance is often due to the presence of multiple efflux pumps and the impermeability of the outer membrane (Poole, 2001, Poole *et al.*, 1993, Ma *et al.*, 1994, Germ *et al.*, 1999, Li *et al.*, 2000). Bacterial porins are water filled channels that are often the major entry point for antibiotics to enter into their intended target (Nikaido, 1992, Hancock, 1987, Nikaido, 2001).

Impermeability of the outer membrane to antibiotics can occur due to mutations in OprD; a *P. aeruginosa* outer membrane porin, which is normally only accessible for small molecules and a few antibiotics, such as β lactams

(Studemeister and Quinn, 1988). These channels close through mutation preventing the entry of antibiotics into the host cell and this leads to the observation of a resistance profile (Studemeister and Quinn, 1988).

P. aeruginosa also harbours a large porin, OprF, which allows for the movement of large compounds such as tetra-saccharides along with the potential movement of antibiotics. Again this entry point can become inhibited which leads to the onset of resistance (Bellido *et al.*, 1992, Hancock and Speert, 2000). *Escherichia coli* (*E. coli*) K-12 *ompF* mutants have been found to be resistant to carbenicillin antibiotics, this shows how bacterial species can alter their phenotypic structures in order to combat the effects imposed upon them by antibiotics (Nikaido, 2003, Harder *et al.*, 1981, Ziervogel and Roux, 2013).

MexAB-OprM is a key efflux pump in *P. aeruginosa* and is involved in the removal of β lactams (Li *et al.*, 1995, Masuda *et al.*, 1999, Nakae *et al.*, 1999), chloramphenicol (Li *et al.*, 1995), fluoroquinolones (Li *et al.*, 1995), sulphonamides (Köhler *et al.*, 1996, Poole, 2001), and trimethoprim (Poole, 2001, Köhler *et al.*, 1996) antibiotics. *P. aeruginosa* also harbours another efflux pump, MexX-MexY, that has the ability to efflux the same molecules as MexAB-OprM but this efflux pump is also involved in the intrinsic resistance of *P. aeruginosa* to aminoglycoside antibiotics (Aires *et al.*, 1999, Westbrock-Wadman *et al.*, 1999, Hancock and Speert, 2000).

Table 4.1: Clinical anti-pseudomonal antibiotics and a description of their functionality, adapted from (Hancock and Speert, 2000). The various *P. aeruginosa* resistance mechanisms are also described for each class of antibiotic emphasising the importance of understanding the underlying mechanisms of antibiotic resistance build up.

Class	Agent(s)	Clinical advantages	Clinical disadvantages	*P. aeruginosa* resistance mechanisms
Penicillin	Ticarcillin Carbenicillin Piperacillin	Synergistic with aminoglycosides used to treat *P. aeruginosa*	May induce β lactamases in *P. aeruginosa*	De-repression of chromosomal β lactamase which allows for the inactivation of β lactams via hydrolysis and so generates increased resistance to other β lactams Overexpression of the MexAB-OprM efflux pump is also detected (Hancock and Speert, 1996)

Cephalosporin	Ceftazidime Cefoperazone Cefepime Cefpirome	Can be used as a single antibiotic treatment method	May induce β lactamases in *P. aeruginosa*	Expression of chromosomal β lactamase which allows for the inactivation of β lactams via hydrolysis and so generates increased resistance to other β lactams Overexpression of the MexAB-OprM efflux pump is also detected (Hancock and Speert, 1996)
Aminoglycoside	Gentamicin Tobramycin	Synergistic with β lactam antibiotics administered to	Narrow therapeutic/toxic ratio	Overexpression of the MexX-MexY pump leading

	Amikacin	treat *P. aeruginosa* infections	Known to penetrate poorly into the cerebrospinal fluid Most large studies have shown that around 10 % of *P. aeruginosa* isolates are resistant to aminoglycosides (Hancock and Speert, 1996)	to *P. aeruginosa* impermeability
Quinolone	Ciprofloxacin	Can be given orally to a patient	Contraindication in children < 16 years	Target site mutations arise in codon 83 of the GyrA/B topoisomerase subunit so altering DNA synthesis levels Overexpression of multiple

				efflux pumps (Mouneimné *et al.*, 1999, Takenouchi *et al.*, 1999)
Polymyxin	Colistin	Very active antibiotics with very little resistance developed thus far	Possible toxicity concerns	Outer lipopolysaccharide changes arise due to regulatory mutations in PhoP/Q however, there is no *in vitro* evidence to document this change (Macfarlane *et al.*, 2000)
Carbapenem (β lactams)	Imipenem Meropenem	Very broad spectrum against a range of Gram negative bacteria Meropenem is not administered as a first line	May induce β lactamases in *P. aeruginosa* Rapid resistance has been seen to develop	Reduction in OprD levels Overexpression of MexAB-OprM efflux pump (when Meropenem is used)

		antibiotic		

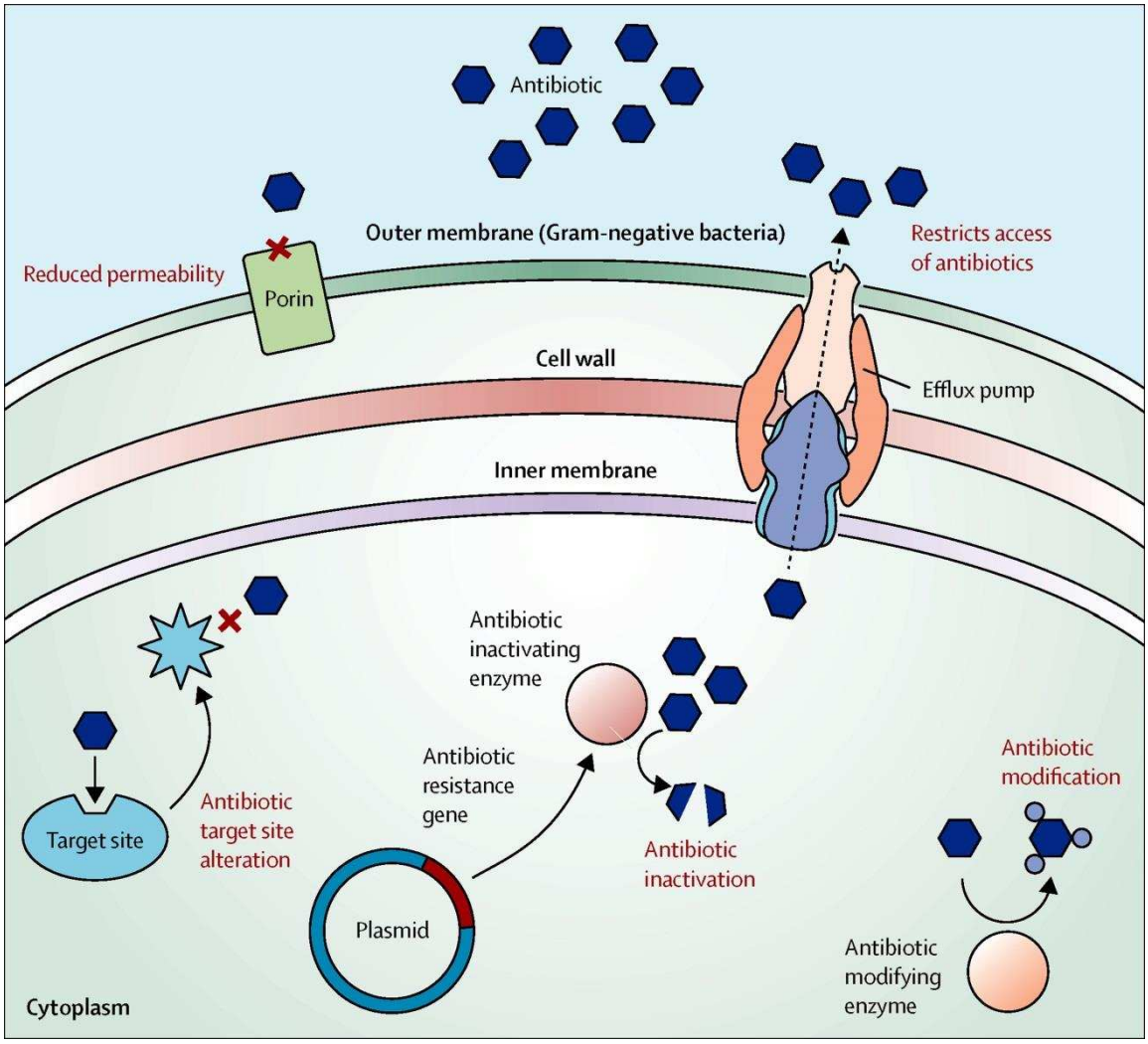


Figure 4.1: Antibiotic resistance mechanisms in Gram negative bacteria (Sherrard *et al.*, 2014). The various antibiotic resistance mechanisms that are present in bacteria isolated from the chronic lung environment such as *Staphylococcus aureus, Burkholderia cepacia* complex C and *P. aeruginosa*.

4.1.1.1. Antibiotic resistance in chronic respiratory disease patients

It has been observed that CF patients clear antibiotics including aminoglycosides and penicillins from their systems faster when compared to healthy individuals (Yaffe *et al.*, 1977, Jusko *et al.*, 1975, Martini *et al.*, 1984, Bosso *et al.*, 1984, Groot *et al.*, 1990, Spino *et al.*, 1984, Touw, 1998, Zobell *et al.*, 2013, Alice S and Neu, 1980). Touw (1998) undertook a review of this elevated clearance rate before concluding that this elevation in penicillin clearance in CF individuals could be potentially be due to CF patients having generally lower body weights leading to lower body surface area ratios, whilst also having increased metabolism rates and increased renal clearance (Yaffe *et al.*, 1977, Jusko *et al.*, 1975, Martini *et al.*, 1984, Bosso *et al.*, 1984, Groot *et al.*, 1990, Spino *et al.*, 1984, Touw, 1998). This elevation in clearance presents clinicians with a challenge delivering the therapeutic dose of antibiotic into the lung (Hutabarat *et al.*, 1991a, Hutabarat *et al.*, 1991b, Levy *et al.*, 1984, Marks, 1981).

To reduce the development of antibiotic resistance, clinicians have started cycling antibiotics. This involves the replacement of a front line antibiotic with a structurally different antibiotic for a period of time, this is done in order to try and reduce the selective pressures which lead to bacterial resistance developing whilst prolonging the efficacy of the front line antibiotic (Bergstrom *et al.*, 2004, Masterton, 2005, Kollef, 2001, McGowan, 1986, Pujol and Gudiol, 2001, Niederman, 1997, Moss *et al.*, 2002). Cycling however, does not provide a long term solution since resistant strains persist in the resident bacterial population and when the initial front line antibiotic is used again, these resistant strains can start to rapidly proliferate (Bergstrom *et al.*, 2004). In order to try to prevent resistance developing towards β lactam antibiotics, antibiotics are often administered with potent inhibitors of β lactamase enzymes. Clavulanic acid is

one of the early inhibitors of β lactamase and combining this inhibitor with a β lactam antibiotic has been successful in clinical settings (Reading and Cole, 1977, Drawz *et al.*, 2014, Shlaes, 2013).

4.1.2. Growth rate alterations of lysogenic bacteria

It has been observed that prophages in *E. coli* strains can contribute towards greater proliferation rates when nutrients are scarce within an environment. This lead to the proposal of a potential beneficial advantage for the incorporation of phages into bacterial chromosomal DNA (Gama *et al.*, 2013, Edlin *et al.*, 1975, Edlin *et al.*, 1977, Lin *et al.*, 1977). This work has previously only been undertaken in laboratory strains of *E. coli* with some very well-studied phages including λ, P1, P2 and Mu (Gama *et al.*, 2013) and it is interesting to consider whether this increase in growth will also be apparent in clinical isolates of *P. aeruginosa* with non-characterised phages.

4.1.3. Type IV pili and twitching motility

Type IV pili are around 6 nm in diameter and normally ~2 µM in length and are primarily located at the poles of the *P. aeruginosa* bacterial cell (Hahn, 1997). The first observation of these appendages on the surface of *P. aeruginosa* was in 1950 by Houwink and van Interson. These appendages are described as either pili or fimbriae (Houwink and Van Iterson, 1950, Weiss, 1971). In *P. aeruginosa*, the main structural subunit is PilA and there are 5 distinct and discrete phylogenetic versions of *pilA*. When characterising the different pili groups of *P. aeruginosa*, there are 5 subgroups as detailed in table 4.2 and visualised in figure 4.2 (Kus *et al.*, 2004). It is believed that this diversity is encoded for by

either random mutations within a strain or through horizontal gene transfer (HGT) occurring with other bacterial species which also harbour pili (Kus *et al.*, 2004).

Each pilin (product of *pilA*) is distinct and can be used for bacterial typing and can be identified by polymerase chain reaction (PCR) (Kus *et al.*, 2004). Downstream of the *pilA* gene is a tRNA site. This region is the target site for primers during PCR amplification and can also be a preferred integration site for bacteriophages (Kus *et al.*, 2004). In 2000, Ochman reviewed the function of tRNA and tRNA-like loci as these loci appear to be common sites for the incorporation of foreign DNA and allow for the formation of many pathogenicity islands (Ochman *et al.*, 2000). $tRNA^{selC}$ has been seen to serve as the integration site for pathogenicity islands in enteric bacteria (Ochman *et al.*, 2000, Ritter *et al.*, 1995). The 70 kb PAI-1 island in uropathogenic *E. coli* integrates at this site (Blum *et al.*, 1994, Ochman *et al.*, 2000) as does the 24 kb SHI-2 island in *Shigella flexneri* (Moss *et al.*, 1999, Vokes *et al.*, 1999, Ochman *et al.*, 2000). Several phages have also been seen to utilise tRNA sites for integration including ϕR73 (Inouye *et al.*, 1991) and P44 of *E. coli*, P22 of *Salmonella* and HPI of *Haemophilus influenzae* (Ochman *et al.*, 2000). The *Streptococcus pyogenes* temperate phage T12 also uses a serine tRNA site in order to insert into its bacterial host cell chromosome (McShan *et al.*, 1997). These studies show the varying uses of the tRNA loci and their importance for pathogenic phage conversions. It is proposed that the integration of phages into the tRNA site within the pilus will have an effect on the diversity or activity of the pilin (Reiter *et al.*, 1989).

Type IV pili can aid bacterial movement on a semi solid surface via a technique called twitching (Semmler *et al.*, 1999, Winstanley *et al.*, 2005, Fonseca *et al.*, 2004, Henrichsen, 1983, O'Toole and Kolter, 1998, Bradley, 1980, LAUTROP, 1961, Mattick, 2002). Twitching occurs as a response to a chemical stimulus and

the bacteria moves via chemotaxis through the extension and retraction of the pilus. Pilus retraction was initially proposed in 1969 to account for the disappearance of type F pili post phage infection and the association of mating cells during conjugation (Marvin and Hohn, 1969, Skerker and Berg, 2001). Subsequent studies have shown that pilus retraction plays a role in the translocation of pilus specific phages in various bacterial host backgrounds including *E. coli*, *P. aeruginosa* and *Caulobacter crescentus* (Skerker and Berg, 2001, Jacobson, 1972, Novotny and Fives-Taylor, 1974, Bradley, 1972a, Bradley and Pitt, 1974, Sommer and Newton, 1988, Skerker and Shapiro, 2000).

It is also possible to hypothesise that the incorporation of a bacteriophage genome into the pilus locus may have an effect on the twitching motility of the *P. aeruginosa* host. Numerous studies have suggested a relationship between phage sensitivity and the presence of functional type IV pili. These studies have also demonstrated that phage resistant mutants are incapable of twitching so linking phage infection and twitching motility (Bradley, 1972a, Bradley, 1972b, Bradley, 1972c, Bradley, 1973c, Bradley, 1973b, Bradley, 1973a, Bradley and Pitt, 1974, Bradley, 1974, Pemberton, 1973, Johnson *et al.*, 1986, Roncero *et al.*, 1990, Whitchurch and Mattick, 1994). It may also be feasible that bacteria lose their type IV pili in order to gain immunity from invading phages. It has been seen that PAO1 harbouring a mutation in the type IV pili biosynthesis genes provides the bacteria with resistance to pilus specific phages (Nunn *et al.*, 1990, O'Toole and Kolter, 1998). To support this statement it has been shown by Kim *et al* (2012) that a lytic phage of *P. aeruginosa*, PA1Ø, utilises the pilus to enter into the *P. aeruginosa* host cell. This shows that it is possible that over time *P. aeruginosa* may lose its pilus in order to provide itself with protection against further phage infections.

Twitching motility is often reduced in CF patients and it has been proposed that this is because there is no evolutionary advantage for the bacterial isolates to retain this function in such a nutrient rich environment (Mahenthiralingam *et al.*, 1994). Earlier isolates have been seen to be motile and express both pili and flagella suggesting that a reduction in motility occurs as CF patients age (Mahenthiralingam *et al.*, 1994).

Table 4.2: The various subgroups of type IV pili of *P. aeruginosa*. Descriptions of the various subgroups are also shown in this table.

Group	Description	Reference
I	Highly conserved, high GC content 51 % and is the most commonly seen subgroup carried by CF *P. aeruginosa* isolates	(Kus *et al.*, 2004, Castric and Deal, 1994)
II	Harbour only the *pilA* gene, no associated accessory genes so is the shortest type IV pili	(Johnson *et al.*, 1986, Pasloske *et al.*, 1985, Sastry *et al.*, 1985, Kus *et al.*, 2004, Castric and Deal, 1994)
III	Contains an open reading frame downstream of *pilA* which encodes for a protein that is homologous to FimB: a pilin accessory protein in *Dichelobacter nodosus* (Gram negative anaerobe)	(Kennan *et al.*, 2001, Kus *et al.*, 2004)
IV	Contains two open reading frames downstream of *pilA* and has a GC content of 54.8	(Kus *et al.*, 2004)

	%	
V	Contains an accessory gene that encodes for a putative protein which shows homology to PilB in *Eikenella corrodens* (Gram negative facultative anaerobic bacillus)	(Villar *et al.*, 1999, Kus *et al.*, 2004)

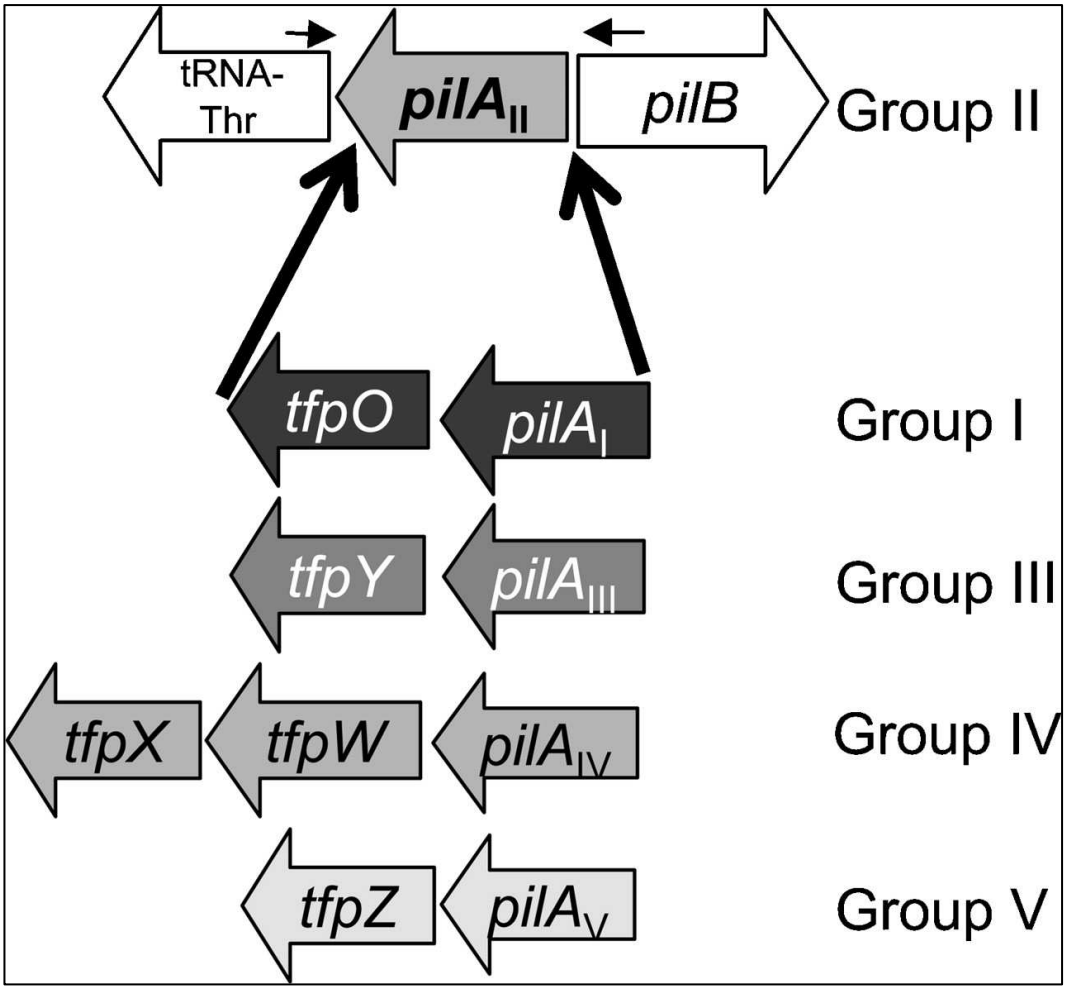


Figure 4.2: The five distinct pilin alleles that are present in *P. aeruginosa* (Kus *et al.*, 2004). Each of the various group's accessory genes are located between the conserved *pilB* and $tRNA^{Thr}$ genes. Table 4.2 details the accessory genes associated within these 5 pilin alleles. Primer locations are represented by the small arrows above the group II image.

4.2. AIMS

The aim of this chapter was to investigate how bacteriophages can pressure twitching motility, growth profiles and antimicrobial resistance of the bacterial hosts. This study again uses the panel of clinical *P. aeruginosa* isolates [CF (n = 47) and BR (n = 47)].

Chapter 3 describes the infection profiles of mixed phage communities whilst in this chapter work begins to focus on how specific bacteriophages influence their bacterial hosts and the bacterium's phenotypic response. To inform the impact of these bacteriophages, singular phage lysogens were created in PAO1. These phages were selected from the panel of *P. aeruginosa* phages due to their ability to re-infect their originating host cell.

These newly created lysogens were then further characterised for *P. aeruginosa* function including antibiotic resistance and growth rates. This chapter focuses mainly on the characterisation of bacterial growth and antimicrobial sensitivity profiles, as it has been demonstrated that phage integration can have a positive effect on bacterial proliferation. It was proposed that the adult CF lysogen would have the most elevated antibiotic resistance profile and growth rate. This proposition is based upon the fact that CF is a lifelong disease and so the *P. aeruginosa* populations will have had to evolve in order to out-compete the other bacterial strains in the lung. However, this isn't always the case and so care is needed when analysing the data presented in this chapter.

Twitching motility assays were undertaken to determine where the mixed viral populations induced from each *P. aeruginosa* isolate in chapter 3, targeted and how this may alter twitching motility. It was hypothesised that the addition of exogenous phage to a bacterial isolate would increase the twitching motility of the isolate. This hypothesis was drawn as it was proposed that the additional

motility which may be generated would be beneficial for both the phage and the *P. aeruginosa* cell in terms of reaching high nutrient areas.

It was also hypothesised that the adult CF phages would be the least motile as it was assumed that this costly function would be lost due to long term colonisation within the chronic lung.

4.3. RESULTS

4.3.1. Purification of bacteriophages and comparison by restriction analysis

The lab strain of *P. aeruginosa*, PAO1, was infected with 4 purified phages using the method described in section 2.3.5. In order to see if there were any genetic differences between the purified phages, the purified phage DNA obtained from each phage was used in restriction digestions (section 2.5.6). The DNA concentration and the 260/280 nm ratios for each phage lysate are detailed in table 4.3.

It was interesting to find that 3 of the 4 isolates had similar genetic fingerprints when subjected to restriction digestion which potentially indicates that this genetic profile is a requirement for host re–infection, digests are shown in figure 4.3. In order to confirm this conclusively however, restriction digestion profiles would need to be compared for phages which do not have the capability to re-infect their originating host. Although this doesn't mean that the diversity is small between these phages, the restriction enzymes used may target areas of the phages that are conserved between phages and thus, banding patterns on the agarose gel may be similar.

Table 4.3: DNA concentrations and 260/280 nm ratios for the different phage lysates. These values were then utilised to set-up the restriction digests.

Purified phage origin	ng/µL	260/230 ratio	260/280 ratio
CF70	176.7	1.90	1.89
CF24	319.4	2.05	1.91
BR123	3000	1.75	1.83
BR136	668.8	1.85	1.91

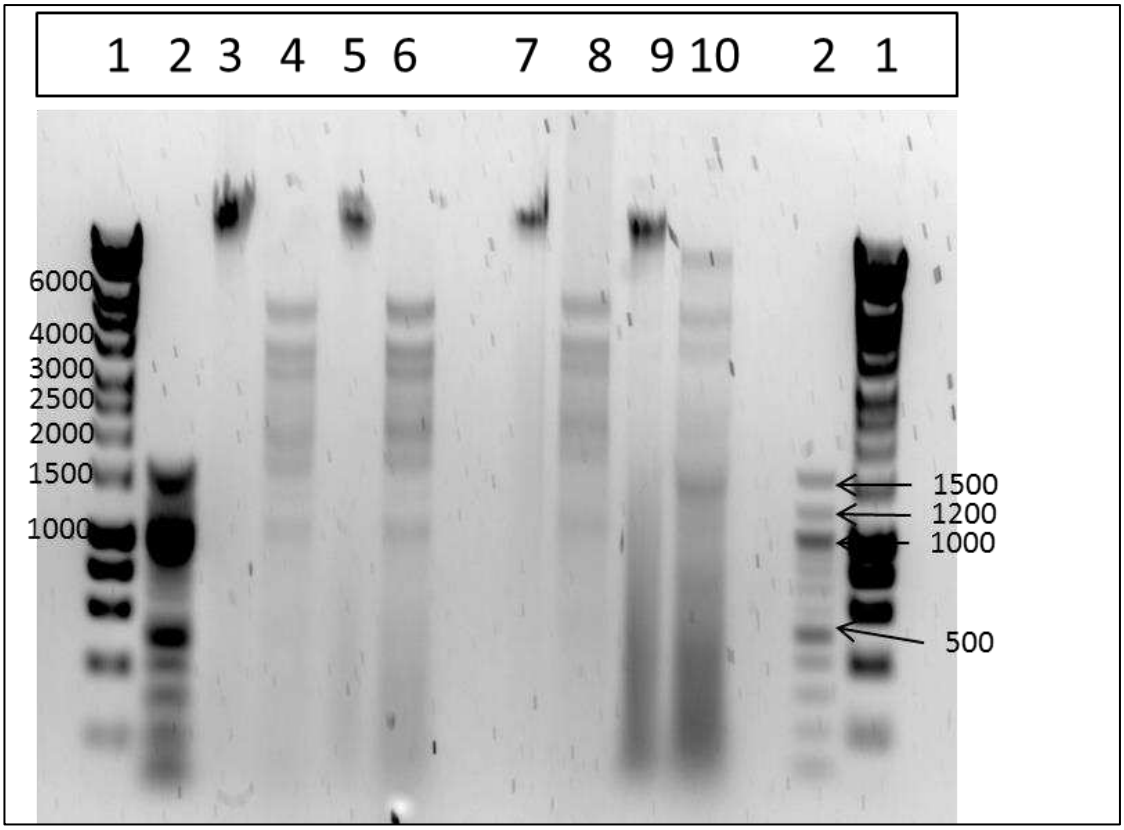


Figure 4.3: Restriction digestion profiles of the four plaque purified phages. A 1 % agarose gel is shown here. Lane 1 – Hyperladder 1 kb, 2 – Quick Load 100 bp ladder, 3 – Uncut 1 µL of ϕ CF24 DNA, 4 – Cut 1 µL ϕ CF24 DNA, 5 – Uncut 3 µL of ϕ CF70 DNA, 6 – Cut 3 µL ϕ CF70 DNA, 7 – Uncut 0.5 µL of ϕ BR123 DNA, 8 – Cut 0.5 µL ϕ BR123 DNA, 9 – Uncut 1 µL of ϕ BR136 DNA, 10 – Cut 1 µL ϕ BR123 DNA

4.3.2. Electron Microscopy images of the plaque purified phages

In order to visualise the plaque-purified phages, electron microscopy images were generated with thanks to the Electron Microscopy unit at Newcastle University, Newcastle upon Tyne, UK (section 2.6). The images provided by the Electron Microscopy unit all had different scale bars so the images which showed the phage morphology the best were chosen to include in this thesis.

These images (figure 4.4) have shown that there is similarity between three of the *P. aeruginosa* phages whilst the fourth has alternative tail morphology. This phage also generates a different banding pattern upon restriction digestion. All the phages shown have a Caudovirales morphology due to long flexible tails being apparent.

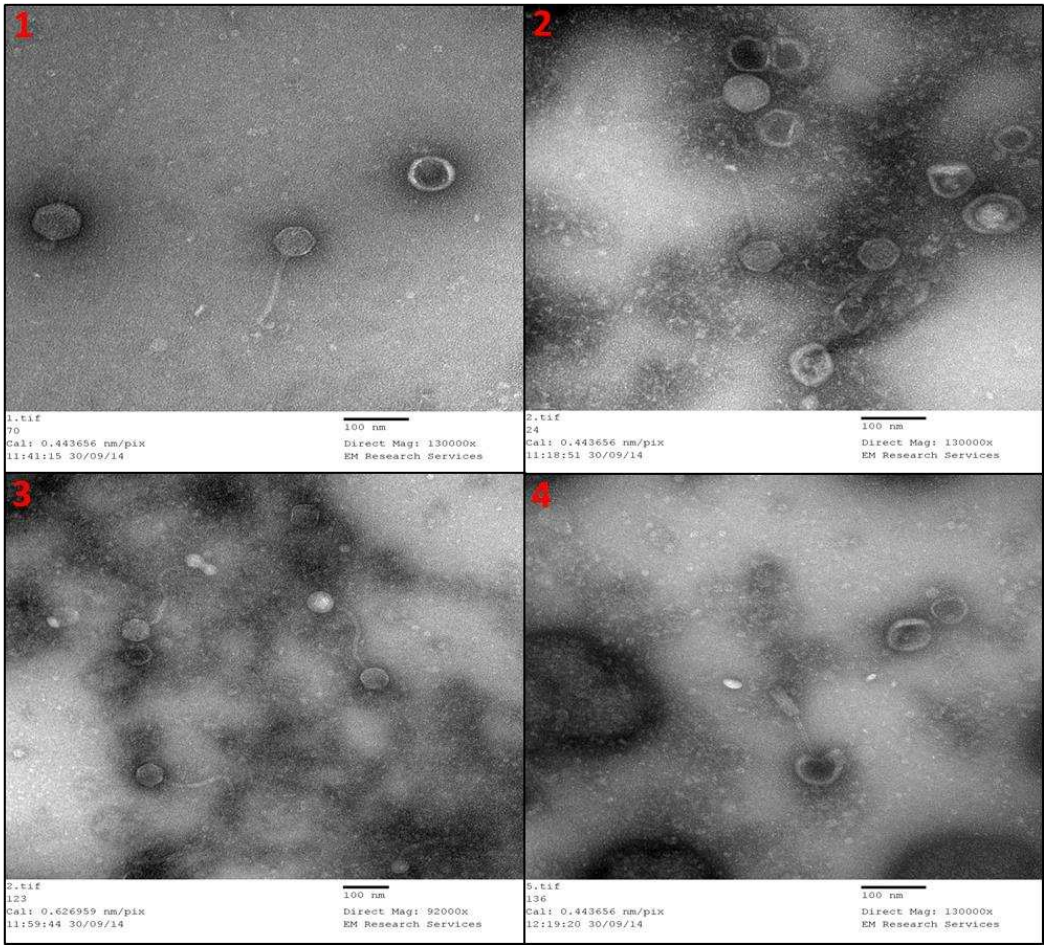


Figure 4.4: Electron microscopy images of the plaque purified phages utilised in this investigation. Panel 1 shows the plaque purified paediatric CF phage, panel 2 shows the plaque purified adult CF phage, panel 3 shows the < 10 BR plaque purified phage whilst panel 4 shows the phage which was plaque purified from a > 10 BR isolate. With thanks to the Electron Microscopy unit at Newcastle University, Newcastle upon Tyne, UK for the generation of these images.

4.3.3. Growth changes of *P. aeruginosa* lysogens compared to naïve PAO1

Upon colonisation of any environment, it is an advantage for a bacterial isolate to localise and out-compete other bacterial species residing within that environment in order for the new bacteria to survive. It has been previously determined that non-clinical *E. coli* strains harbouring a prophage exhibit an elevated growth rate compared to non-lysogenic strains (Edlin *et al.*, 1977, Edlin *et al.*, 1975, Lin *et al.*, 1977).

Comparative growth curves were used to compare each of the purified phage lysogens versus the uninfected PAO1. Figure 4.5 shows the growth rates for each lysogenic bacterium compared to the non-lysogenic PAO1. In order to determine that the increase in absorbance detected was due to an elevation in cell count rather than an accumulation of cell debris, the cfu/mL for each lysogen was determined at 9 hours and 18 hours, as shown in figure 4.6. All the associated raw data is shown in appendix 4.

The lysogens demonstrated an elevated growth rate compared to the naïve PAO1 in terms of growth over an 18 hour growth period with the exception of BR123 however, this growth rate was not always statistically significant. These lysogens showing an elevation in growth supports previous findings in *E. coli* (Edlin *et al.*, 1977, Edlin *et al.*, 1975, Lin *et al.*, 1977). These previous studies were undertaken in non-clinical isolates and also under nutrient starvation whilst here clinical isolates were utilised and the lysogens grown in nutrient rich conditions.

It was seen that the most significant differences between non-lysogenic and lysogenic growth was determined for the adult CF lysogen; CF24. This potentially is due to the adult CF phages being a long term coloniser of the lung and thus,

has adapted numerous methods in order to provide its bacterial host with a selective advantage and allow for the maintenance of both entities within this community. It is also a possibility that this phage is just more adapted to its bacterial host and the disease state of the patient is irrelevant to the phages functionality within this experiment. The > 10 BR lysogen (BR136) showed similar growth patterns as the non-lysogenic PAO1 until around 4.30 hours of growth after this the lysogen showed an elevation in growth compared to the control.

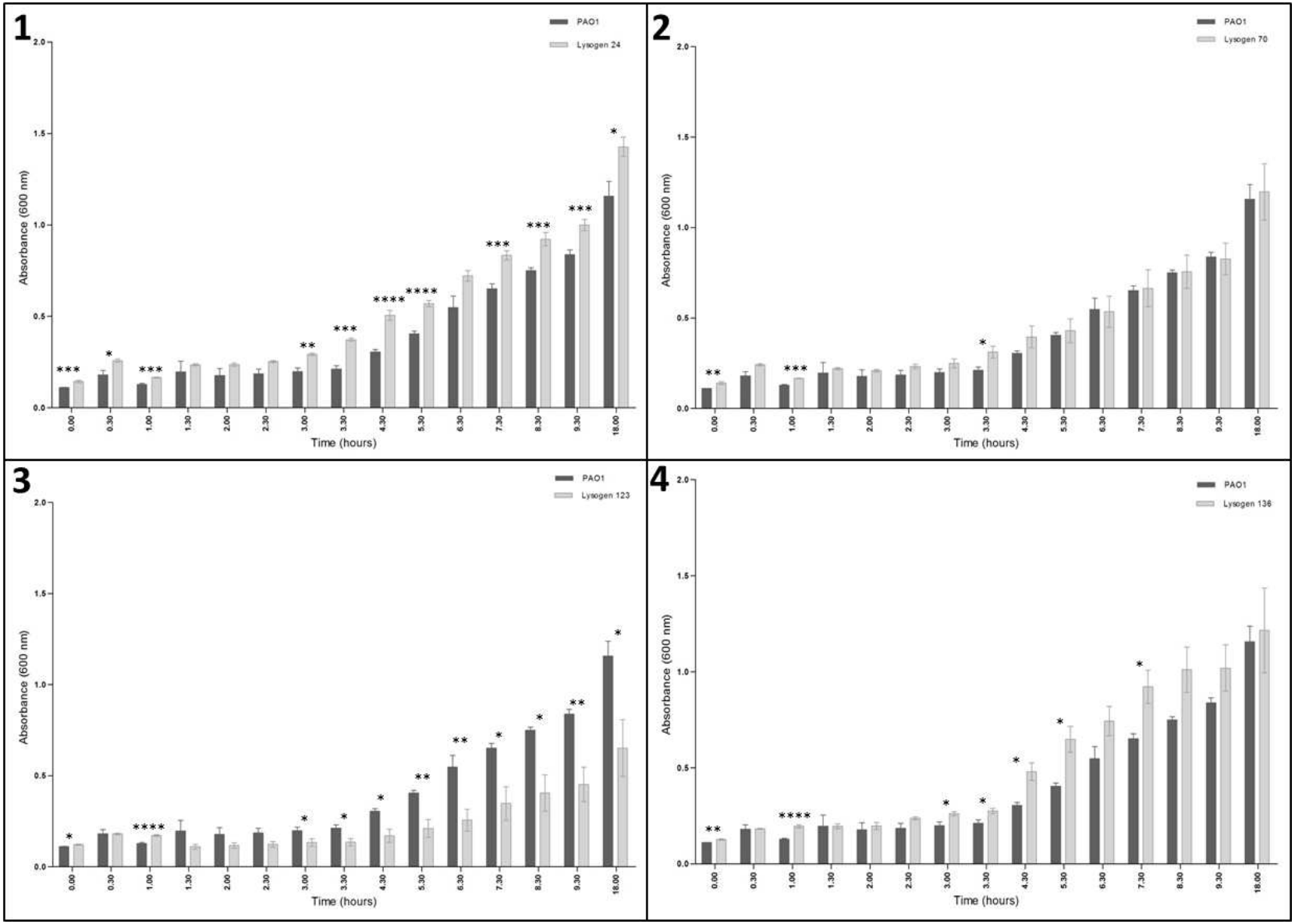

1
PAO1
Lysogen 24
Absorbance (600 nm)
Time (hours)
2
PAO1
Lysogen 70
Absorbance (600 nm)
Time (hours)
3
PAO1
Lysogen 123
Absorbance (600 nm)
Time (hours)
4
PAO1
Lysogen 136
Absorbance (600 nm)
Time (hours)

Figure 4.5: Comparing growth rates for lysogenic bacteria compared to naïve non-lysogenic bacteria. Growth rate comparisons of the lysogens compared to the naïve PAO1 host, absorbance is determined at 600 nm and growth was recorded over an 18 hour period (n = 15). Non parametric t tests were utilised with two tailed p values shown in order to display the most discrete statistical significant data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001.

Panel 1 shows the growth rate changes of the adult CF lysogen (CF24) compared to naïve PAO1 over an 18 hour period.

Panel 2 shows the growth rate changes of the paediatric CF lysogen (CF70) compared to naïve PAO1 over an 18 hour period.

Panel 3 shows the growth rate changes of the < 10 BR lysogen (BR123) compared to naïve PAO1 over an 18 hour period.

Panel 4 shows the growth rate changes of the > 10 BR lysogen (BR136) compared to naïve PAO1 over an 18 hour period.

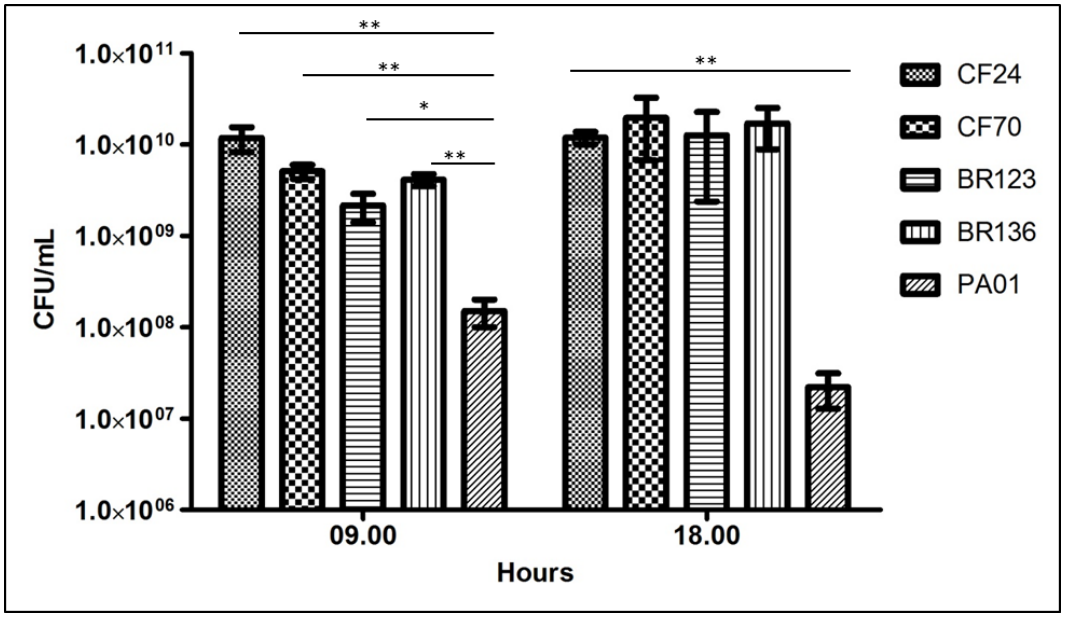


Figure 4.6: Validation that the growth curve absorption results are due to cell growth and not a build-up of cell debris. Non parametric t tests were utilised with two tailed p values shown in order to display the most discrete statistical significant data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001 (n = 15).

Cell counts were done for the 4 lysogens and naïve PAO1 after growth for 9 and 18 hours in order to validate the observations that the increase in absorbance was due to elevated bacterial growth rather than a build-up of cellular debris which would affect the absorbance readings.

4.3.4. Changes to antibiotic sensitivity to clinically relevant anti-pseudomonal antimicrobials

4.3.4.1. Minimum Inhibitory concentration (MIC) calculation

Differences in the antibiotic sensitivity profiles between the purified phage lysogens and the naïve PAO1 were investigated. The lysogens were subjected to MIC assays using clinically relevant anti-pseudomonal antibiotics, as described in methods section 2.4.1. Untreated PAO1 was used as a growth control and determined basal resistance. The raw data values are shown in appendix 5.

The MIC assay results are shown in figure 4.7, section 1. The MIC assays were based upon absorbance changes (OD_{600}) with an elevated absorbance indicating an increase in the number of bacteria surviving. The results post 9 hours of antibiotic treatment are shown.

4.3.4.2. Normalisation for bacterial growth rate

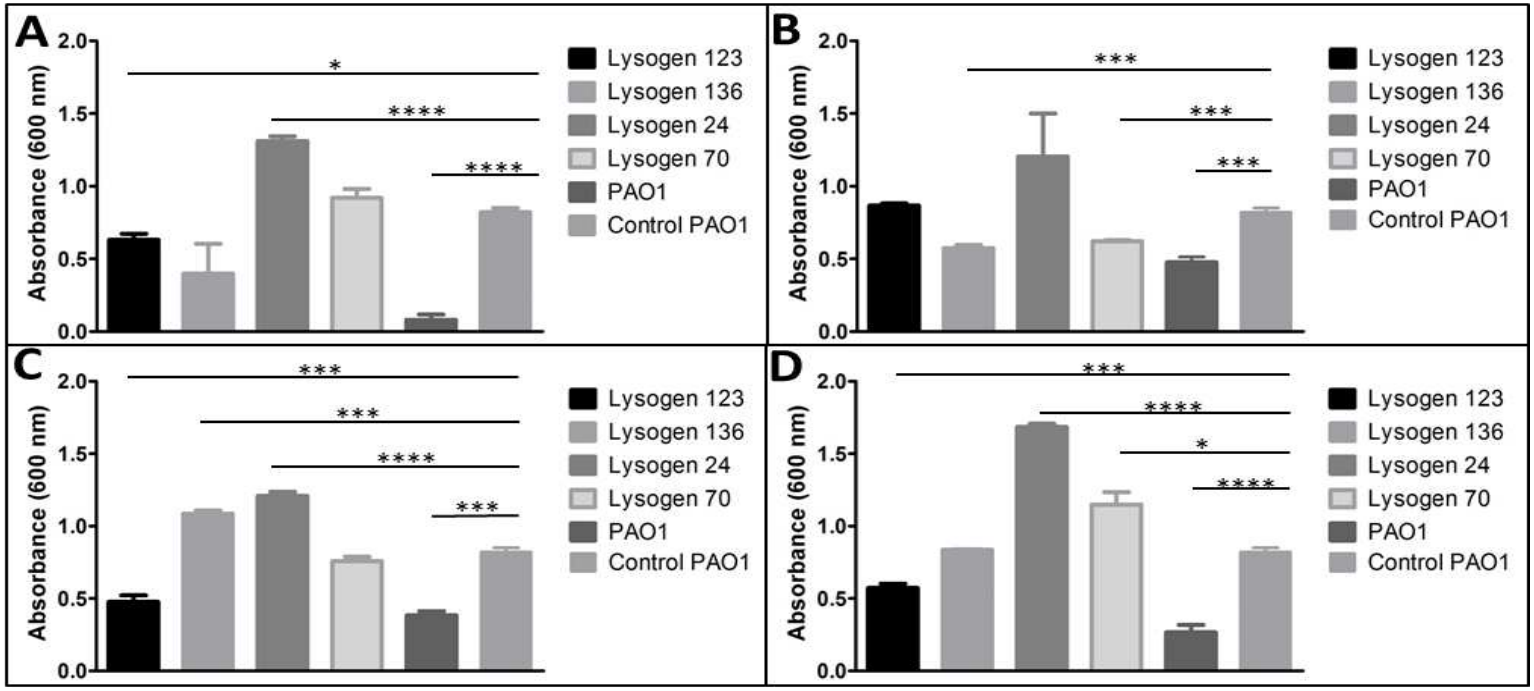
Growth differences were also taken into account by normalising the results against the growth rates of the bacteria over the same time period but without antibiotic treatment. This was completed in order to calculate the fold change in growth in the presence of an inhibitory concentration of the drug, this allowed conclusions to be drawn to determine whether the increase in antibiotic tolerance was in fact due to elevated bacterial growth or whether it was related to the prophage or a prophage encoded process; figure 4.7, section 2. In order to confirm that the increase in absorbance was not due to an increase in cell debris which could be generated by lytic phage induction, cell counts and plaque assays were undertaken (table 4.4).

The lysogens had elevated resistance after 9 hours of growth compared to PAO1 treated with the same amount of antibiotic. Naïve PAO1 with and without antibiotic treatment was included as a control. These results indicate that the prophage is providing the lysogen with a beneficial advantage for survival in an environment that is often treated with large numbers of antibiotics.

In accordance with the hypothesis initially proposed, it would appear that the lysogen containing the phage originating from the adult CF *P. aeruginosa* isolate had an elevated tolerance to antibiotic treatment. It was observed upon normalisation for growth, that the lysogens derived from CF *P. aeruginosa* isolates had the lowest change in growth. The change in bacterial growth and antibiotic resistance was higher for the lysogens with phages originating from BR *P. aeruginosa* isolates. These data led to the assumption that the BR and CF phages are functioning via different mechanisms in order to provide their bacterial hosts with an elevated antimicrobial tolerance profile. These mechanistic differences may be representative of the fact that the phages have had altered lengths of time to colonise the chronic lung. Potentially the more naïve BR phages may not be conferring as great a selective advantage to their *P. aeruginosa* host compared to the evolved CF phages. Disease longevity is not an indication of *P. aeruginosa* colonisation time though.

These results were generated via the use of one phage from each clinical aetiological subgroup, this means that care needs to be taken when analysing the data so that no presumptions are drawn relating to patient health and evolution/adaptation within the CF/BR lung.

1



2

Antibiotic	Lysogen 24	Lysogen 70	Lysogen 123	Lysogen 136
Ceftazidime	1.34	0.72	1.77	1.94
Colistin	4.37	2.16	3.46	4.12
Meropenem	2.89	1.60	3.65	1.68
Piperacillin	1.38	0.22	2.56	1.79

Figure 4.7: Variation in antibiotic tolerance when comparing lysogenic bacteria to naïve non-lysogenic bacteria. Section 1 shows the alteration in the antibiotic sensitivity profiles for the lysogens when treated with Ceftazidime (0.08 µg/mL), Colistin (1.6 µg/mL), Meropenem (0.08 µg/mL) or Piperacillin (0.8 µg/mL); n = 6. The changes are observed as an increase in absorbance (OD_{600}) over a 9 hour period. Non parametric t tests were utilised with two tailed p values shown in order to display the most discrete statistical significant data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001. All the antibiotics were used at 1,000th of the MIC for each antibiotic and *P. aeruginosa* (BASC data – accessed 16.07.16).

Panel A shows the change in absorbance (OD_{600}) for the lysogen compared to naïve PAO1 growth when treated with 0.08 µg/mL ceftazidime.

Panel B shows the change in absorbance (OD_{600}) for the lysogen compared to naïve PAO1 growth when treated with 1.6 µg/mL colistin.

Panel C shows the change in absorbance (OD_{600}) for the lysogen compared to naïve PAO1 growth when treated with 0.08 µg/mL meropenem.

Panel D shows the change in absorbance (OD_{600}) for the lysogen compared to naïve PAO1 growth when treated with 0.8 µg/mL piperacillin.

Section 2 details fold increase changes for each lysogen upon normalisation for lysogen growth.

Table 4.4: Cfu and Pfu calculations to show the results are due to lysogenic growth rather than an increase in cellular debris. The Pfu shows the free phage is present after 9 hours of growth and the cfu of the bacteria after 9 hours of growth was also determined. These values were calculated in order to show that the results seen are due to lysogenic conversion of the bacterial host rather than an increase in cell debris through lytic phage growth. Ceftazidime, Meropenem and Piperacillin targeted peptidoglycan synthesis whilst colistin targets the cytoplasmic membrane, the altered targets may affect the production of free phage. Colistin generated no detectable pfu counts post 9 hours of incubation so indicating that there may be no free phage present and that complete lysogenic conversion has occurred within these lysogenic bacteria. The different target of colistin may also be indicative as to why there is no discernible free phage present within the assays. Naïve PAO1 at the same time point had a Cfu value of 1.5 $x10^{08}$ which is lower than the cfu values shown for the lysogens post antibiotic treatment.

Antibiotic	Lysogen	Pfu	Cfu
Ceftazidime	24	5.60×10^{10}	3.25×10^{09}
	70	9.07×10^{10}	5.00×10^{09}
	123	3.47×10^{11}	8.50×10^{08}
	136	2.25×10^{11}	3.00×10^{09}
Meropenem	24	5.26×10^{11}	4.25×10^{09}
	70	7.55×10^{11}	1.00×10^{09}

	123	2.58×10^{10}	4.50×10^{08}
	136	6.07×10^{11}	3.00×10^{09}
Piperacillin	24	2.76×10^{11}	8.58×10^{08}
	70	2.68×10^{11}	3.00×10^{09}
	123	2.94×10^{11}	4.25×10^{09}
	136	1.68×10^{11}	1.00×10^{09}

4.3.5. Mixed phage lysates impact on twitching motility of the clinical *P. aeruginosa*

Twitching motility as previously described (section 4.1.3) is a biological function of *P. aeruginosa* which is utilised to move the bacterium along chemical gradients. Twitching of *P. aeruginosa* has previously been shown to lost with adaptation to the CF lung (Mahenthiralingam *et al.*, 1994). Type IV pili have also been shown to be a preferential adsorption and chromosomal integration site for phages due to the presence of a tRNA gene (Ochman *et al.*, 2000, Inouye *et al.*, 1991, McShan *et al.*, 1997). Using the mixed phage communities induced from the individual *P. aeruginosa* isolates, it was possible to see if these phages altered the ability of the bacterium to twitch subsequent to infection. Raw data is described in appendix 6.

Twitching motility assays were undertaken to ascertain the interaction and impact of phages, see methods section 2.4.4. Differences in twitching may correlate to the host range differences seen in the phage infectivity and bacterial sensitivity studies or may indicate whether a specific subtype is linked to this difference.

The range and distribution of the twitching motility results and the median values are shown in table 4.5. In order to conclude whether the bacterial isolates were motile, LESB58 was used as a negative control as it is known not to twitch (Winstanley *et al.*, 2009). Any isolate that exhibited a twitching profile that was greater than LESB58 was considered motile, shown in figure 4.8.

These data generated show that the adult CF *P. aeruginosa* isolates did have reduced twitching in comparison to the BR isolates, so supporting previously drawn hypotheses about motility being lost during long term *P. aeruginosa* colonisation (Mahenthiralingam *et al.*, 1994). These results also show that the

adult CF *P. aeruginosa* isolates had elevated motility when comparing them against the paediatric CF *P. aeruginosa* isolates.

Twitching was seen to increase in some clinical *P. aeruginosa* isolates upon the addition of self-induced phage, indicating that the presence or induction of phages may have an evolutionary advantage for the host cell in reaching areas of high nutrient availability via chemotaxis. However, twitching was seen to decrease in most instances upon addition of self-induced phage, so showing the variability in bacterial-phage infections whilst also emphasising the complexity of interactions occurring within the CF/BR lung.

3 paediatric CF *P. aeruginosa* isolates showed an elevation in twitching with the addition of exogenous phages and 5 adult CF *P. aeruginosa* isolates also showed this elevation. 4 < 10 BR *P. aeruginosa* isolates exhibited elevated twitching when exogenous self-induced phage was added into the twitching motility assay and 2 > 10 BR *P. aeruginosa* isolates also showed this elevation in motility. However, when comparing against the cut-off value for LESB58, none of the 3 paediatric CF isolates could be classed as motile but all 5 of the adult CF isolates could. Only 1 of the 3 < 10 BR *P. aeruginosa* isolates could be classed as motile but the 2 > 10 BR *P. aeruginosa* isolates could be classified as motile. In some instances, it was observed that the addition of exogenous phage caused a reduction in the twitching motility of the bacterium, indicating that these phages must be providing their hosts with a disadvantage compared to naïve, non-phage infected isolates.

Table 4.5: Range in the distribution seen in the twitching assays with and without the addition of self-induced phage. The median values are also included and they show the medians for twitching are higher in the BR *P. aeruginosa* isolates compared to the CF isolates with and without the addition of self-induced phage. This supports previous work that shows that the ability to twitch is lost in CF isolates.

***P. aeruginosa* isolate**	**Range of distribution with added phage (mm)**	**Median value (mm)**	
Paediatric CF	3 – 26	5	**With added phage**
Adult CF	2 – 24	5	
< 10 BR	3 – 21	5	
> 10 BR	3 – 20	10	
Paediatric CF	3 – 27	6	**Without added phage**
Adult CF	3 – 32	6	
< 10 BR	3 – 21	7	
> 10 BR	5-26	11	

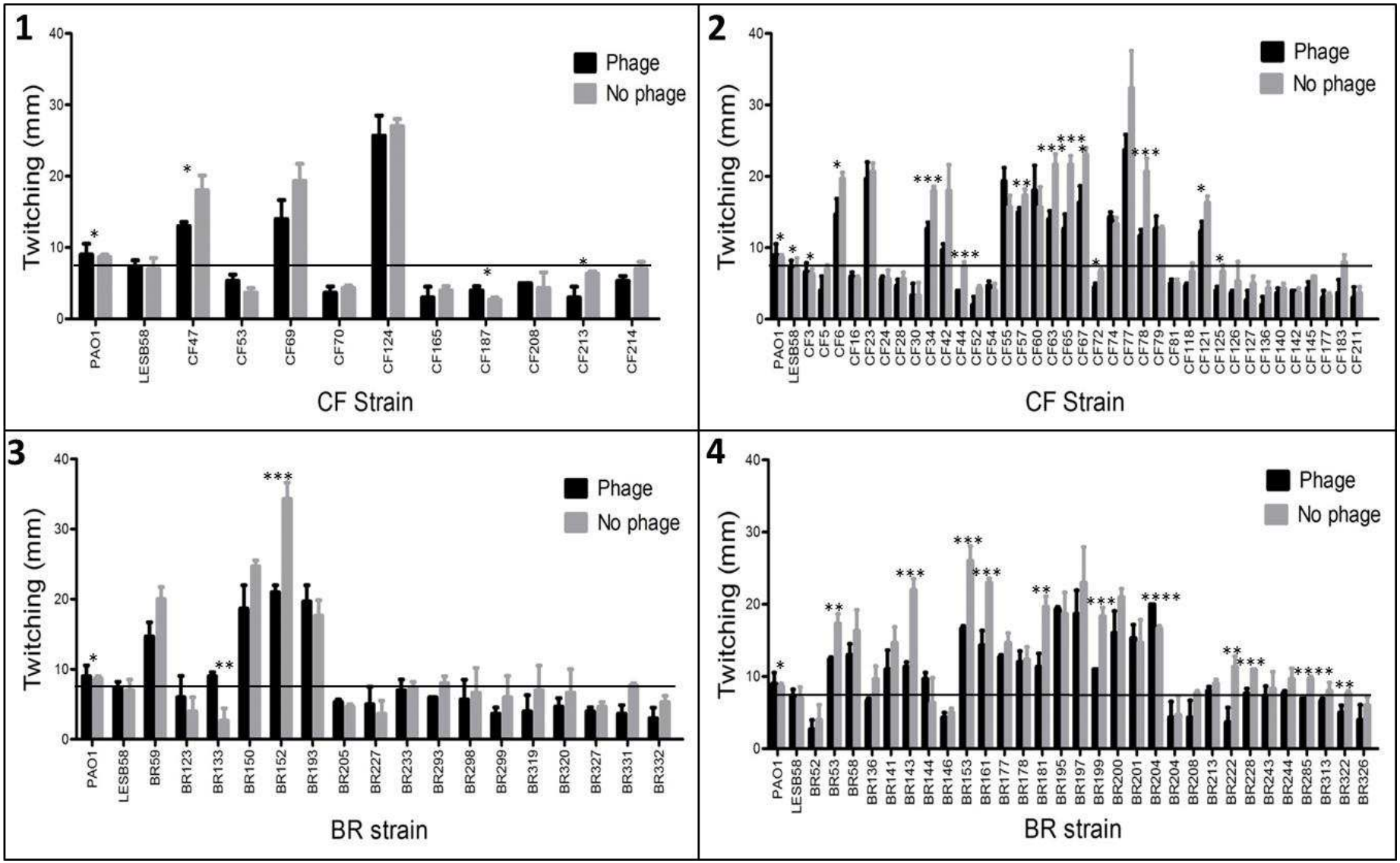

1
Twitching (mm)
Phage
No phage
PAO1
LESB58
CF47
CF53
CF69
CF70
CF124
CF165
CF187
CF208
CF213
CF214
CF Strain
2
Twitching (mm)
Phage
No phage
CF Strain
3
Twitching (mm)
Phage
No phage
PAO1
LESB58
BR59
BR123
BR133
BR150
BR152
BR193
BR205
BR227
BR233
BR293
BR298
BR299
BR319
BR320
BR327
BR331
BR332
BR strain
4
Twitching (mm)
Phage
No phage
BR strain

Figure 4.8: Twitching motility of clinical *P. aeruginosa* from each aetiological group with addition of self-induced mixed phage lysates compared to no phage. PAO1 was included as the positive control as it is known to contain type IV pili and so have the ability to twitch, LESB58 was included as a negative control. The line indicates strains that have elevated motility in respect to LESB58 and so in this context these strains are classed as motile; n = 3. Non parametric t tests were utilised in order to analyse this data and two tailed p values are shown on these data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001.

Panel 1 is the paediatric CF *P. aeruginosa* strains with and without the addition of mixed phages.

Panel 2 is the adult CF *P. aeruginosa* strains with and without the addition of mixed phages.

Panel 3 is the < 10 BR *P. aeruginosa* strains with and without the addition of mixed phages.

Panel 4 is the > 10 BR *P. aeruginosa* stains with and without the addition of mixed phages.

4.3.6. PCR characterisation of clinical *P. aeruginosa* isolates for the type IV twitching pili

PCR of the pili was undertaken in order to determine which pilin subgroup was harboured by the *P. aeruginosa* isolates and to see if there some isolates which harboured no detectable pili via PCR. Type IV pili of *P. aeruginosa* may be detectable via PCR but this observation does not always indicate that *P. aeruginosa* isolates are motile (Kus *et al.*, 2004). Type IV pili may be present on the *P. aeruginosa* isolate but are non–motile due to a defect in one of the pilus genes or potentially as a host immunity defence mechanism (Kus *et al.*, 2004). Due to the similarity in band sizes for the different subgroups of the type IV pili, this work did not allow for observation of the discrete sub-groups. If it was possible to separate the PCR results into subgroups then according to Kus *et al* (2004); group II pili would generate a 1.4 kb product, group I or III would generate a 3 kb product, group I, III or V would generate a 2.5 kb product whilst a group IV pili would generate a 4 kb product.

The PCR images for the various aetiologies are shown in figures 4.9 – 4.12. Identification of a PCR product allowed for the observation of the number of isolates from each of the clinical aetiologies which had no detectable pili: : 10 % of paediatric CF *P. aeruginosa* isolates, 24 % of adult CF *P. aeruginosa* isolates, 23 % of < 10 BR *P. aeruginosa* isolates and 30 % of > 10 BR *P. aeruginosa* isolates. It is however, pertinent to remember differences in the cohort sizes when analysing these results. It was observed that some *P. aeruginosa* isolates generated more than one PCR product but this maybe a consequence of these *P. aeruginosa* isolates harbouring only a single flagellum but multiple pili (Skerker and Berg, 2001). Type IV pili have been seen to form bundles on the poles of cyanobacteria (Gram negative bacterium that has originated from an aquatic

environment), so it is possible to propose that this observation could also be apparent in *P. aeruginosa* isolates (Schuergers and Wilde, 2015).

From the 10 % of paediatric *P. aeruginosa* isolates which have detectable pili 4 can positively twitch and from the 24 % of adult *P. aeruginosa* isolates with detectable pili 12 isolates exhibit positive twitching. The data from without the addition of self-induced phage is used to generate these data as it is perceived to be more accurate and reproduce more accurate real-life results. 23 % of the < 10 BR *P. aeruginosa* isolates have detectable type IV pili as determined by PCR and 7 of these strains exhibit positive twitching. 30 % of the > 10 BR *P. aeruginosa* isolates have detectable type IV pili and 11 could positively twitch. This would indicate that for some strains the type IV pili are non-functional as no positive twitching was recorded even though a product was generated via PCR. All the strains with detectable pili were sensitive to phage infection and so this may indicate that the phages in this thesis are utilising the pilus as a receptor to aid host infection.

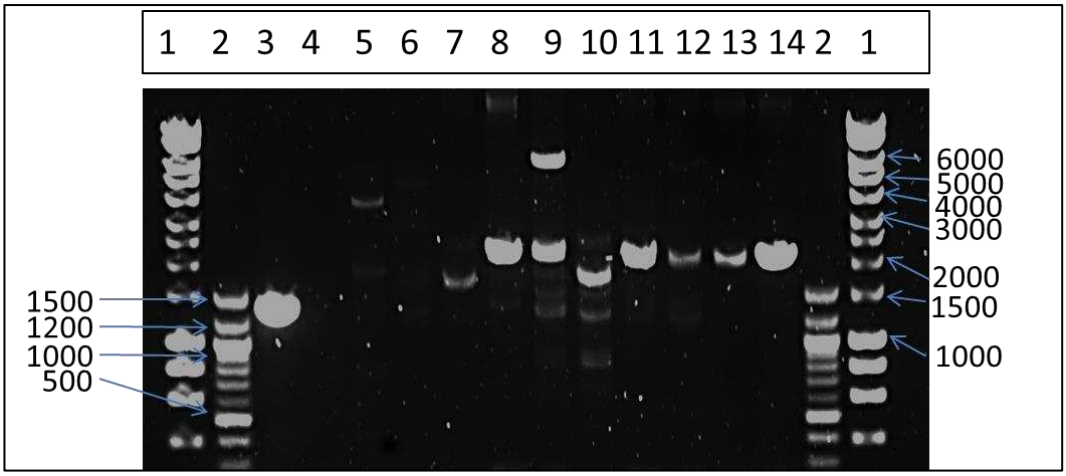


Figure 4.9: Presence of type IV pili in paediatric CF isolates. 1 % agarose gel showing the PCR products for the paediatric CF *P. aeruginosa* isolates, the ladder sizes are shown on the gel. Lane 1 – Hyperladder 1 kb, lane 2 – Quick Load 100 bp ladder, lane 3 – Positive PAO1, lane 4 – Negative, lane 5 – CF47, lane 6 – CF53, lane 7 – CF69, lane 8 – CF70, lane 9 – CF124, lane 10 – CF165, lane 11 – CF187, lane 12 – CF208, lane 13 – CF213 and lane 14 – CF214

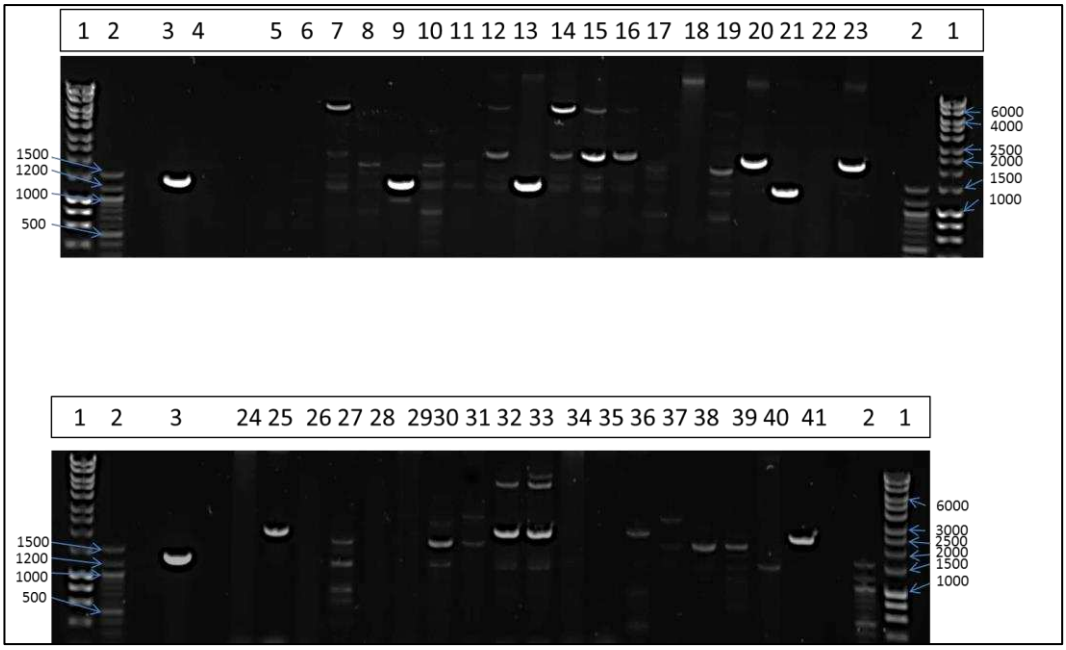


Figure 4.10: Presence of type IV pili in adult CF isolates. 1 % agarose gel showing the PCR products for the adult CF *P. aeruginosa* isolates, the ladder sizes are shown on the gel. Lane 1 – Hyperladder 1kb, lane 2 – Quick load 100 bp ladder, lane 3 – Positive PAO1, lane 4 – Negative, lane 5 – CF3, lane 6 – CF5, lane 7 – CF6, lane 8 – CF16, lane 9 – CF23, lane 10 – CF24, lane 11 – CF28, lane 12 – CF30, lane 13 – CF34, lane 14 – CF42, lane 15 – CF44, lane 16 – CF52, lane 17 – CF54, lane 18 – CF55, lane 19 – CF57, lane 20 – CF60, lane 21 – CF63, lane 22 – CF65, lane 23 - CF67, lane 24 – CF72, lane 25 – CF74, lane 26 – CF77, lane 27 – CF78, lane 28 – CF79, lane 29 – CF81, lane 30 – CF118, lane 31 – CF121, lane 32 – CF125, lane 33 – CF126, lane 34 – CF127, lane 35 – CF136, lane 36 – CF140, lane 37 – CF142, lane 38 – CF145, lane 39 - CF177, lane 40 – CF183 and lane 41 – CF211

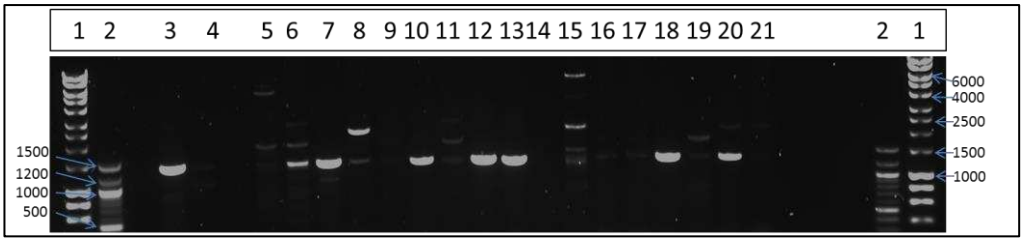


Figure 4.11: Presence of type IV pili in < 10 BR isolates. 1 % agarose gel showing the PCR products for the < 10 BR *P. aeruginosa* isolates, the ladder sizes are shown on the gel. Lane 1 – Hyperladder 1 kb, lane 2 – Quick Load 100 bp ladder, lane 3 – Positive PAO1, lane 4 – Negative, lane 5 – BR59, lane 6 – BR123, lane 7 – BR133, lane 8 – BR150, lane 9 – BR152, lane 10 – BR193, lane 11 – BR206, lane 12 – BR227, lane 13 – BR233, lane 14 – BR293, lane 15 – BR298, lane 16 – BR299, lane 17 – BR319, lane 18 – BR320, lane 19 – BR327, lane 20 – BR331 and lane 21 – BR332

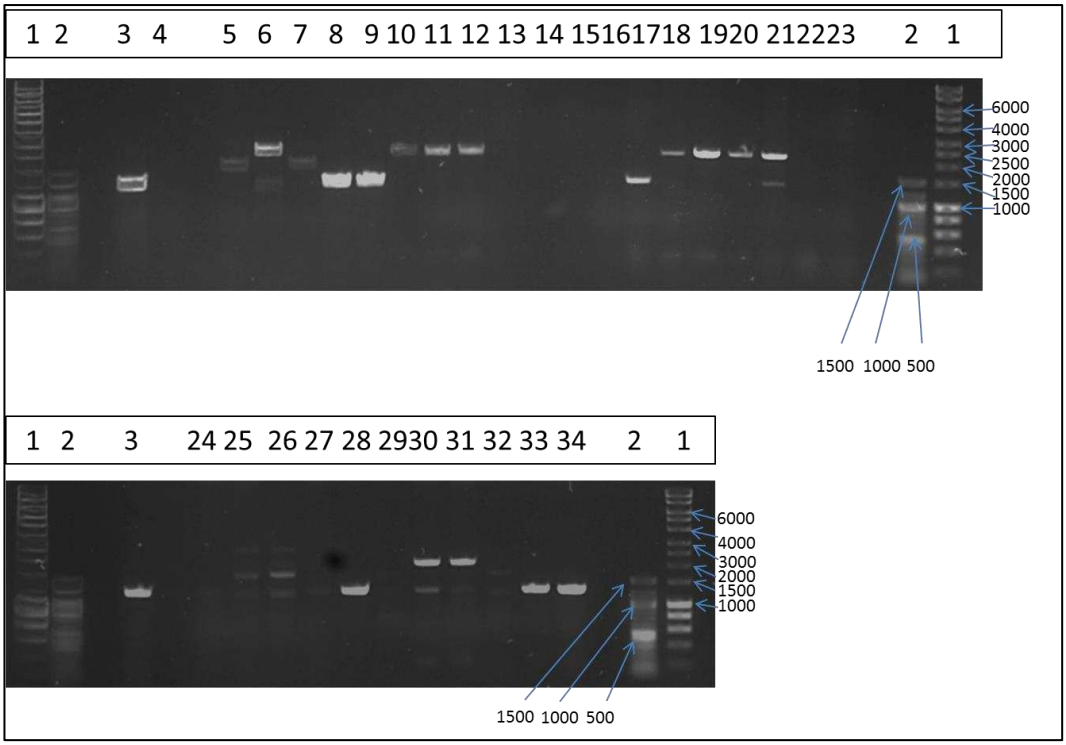


Figure 4.12: Presence of type IV pili in > 10 BR isolates. 1 % agarose gel showing the PCR products for the > 10 BR *P. aeruginosa* isolates, the ladder sizes are shown on the gel. Lane 1 – Hyperladder 1 kb, lane 2 – Quick Load 100 bp ladder, lane 3 – Positive PAO1, lane 4 – Negative, lane 5 – BR52, lane 6 – BR53, lane 7 – BR58, lane 8 – BR136, lane 9 – BR141, lane 10 – BR143, lane 11 – BR144, lane 12 – BR146, lane 13 – BR153, lane 14 – BR161, lane 15 – BR177, lane 16 – BR178, lane 17 – BR181, lane 18 – BR195, lane 19 – BR197, lane 20 – BR199, lane 21 – BR200, lane 22 – BR201, lane 23 – BR204, lane 24 – BR205, lane 25 – BR208, lane 26 – BR213, lane 27 – BR222, lane 28 – BR228, lane 29 – BR243, lane 30 – BR244, lane 31 – BR285, lane 32 – BR313, lane 33 – BR322 and lane 34 – BR326

4.4. DISCUSSION

Phages are increasingly being linked to adaptation and aiding the evolution of bacteria in environmental niches. Several studies have determined that phages carry a high number of small accessory genes with no known function which could potentially be driving this adaptation and evolution (Smith *et al.*, 2007). These genes are however, conserved throughout their specific phage types and thus, must play a role in the phages survival. This statement can be supported by the observation of mosaic segments in the genomes of unrelated phages of *E. coli* and *Salmonella*. Phage λ for example was isolated in 1951 as a prophage in an *E. coli* K12 isolated from a Californian patient (Lederberg, 1951, Sanger *et al.*, 1982, Juhala *et al.*, 2000). The same mosaic genetic sequence was also detected in a phage of *Salmonella typhimurium*, P22, which was identified in 1952 in either Sweden or Chile (Zinder and Lederberg, 1952, Lilleengen, 1984, Juhala *et al.*, 2000). Finally, the same mosaic sequence was detected in HK97 and HK022, phages of *E. coli* which were identified in 1975 in Hong Kong (Dhillon *et al.*, 1980, Dhillon and Dhillon, 1976, Juhala *et al.*, 2000). These findings show the conserved nature of certain genome regions between phages and led to the proposition that their importance for the phage is shown by their conserved nature.

The majority of the work detailed in chapter 3 involved the use of mixed temperate bacteriophage communities in order to provide a general view of the relationship between phages and bacteria. The work detailed in this current chapter has described the possible evolutionary advantages for *P. aeruginosa* to harbour temperate phages. In order to allow for more detailed work to be undertaken, proposed well adapted phages were isolated from a subset of isolates via plaque purification. The bacteriophages were perceived to be well adapted due to their ability to re-infect their originating host bacterium, this is

considered advantageous as it means that the temperate bacteriophage always has a bacterium into which it can integrate and form a prophage. Restriction digestion showed that 3 out of the 4 induced phages had similar genetic profiles. This is not unexpected however, as the 4 *P. aeruginosa* isolates were originally selected due to their ability to re-infect their originating host.

Another possible reason for this similarity could be because restriction enzymes which target conserved sites within the phage genome were selected. These enzymes may therefore, produce profiles that offer similar banding patterns. We can say though that due to the differences observed in the growth and antimicrobial tolerance profiles, these phages are genetically different in some way. If this were to be repeated, the use of pulse field electrophoresis would be used to offer better definition of the phage restriction profiles. To overcome this, these phage and lysogen genomes are currently being sequenced and annotated for publication but due to time constraints these are not included in this thesis.

The plaque purified phages were imaged through the use of electron microscopy and it was observed that all the phages had a *Caudovirales* morphology. This is not uncommon as in 2010 it was proposed that 97 % of all the known phages of *P. aeruginosa* harboured this morphology (Ceyssens and Lavigne, 2010). It is interesting to note that the tail fibre of the > 10 BR phage is different to the other 3 purified phages, this adaptation may be due to an altered cell surface target for this phage for example. This phage also generated an alternative banding pattern upon restriction digestion potentially indicating that this phage has an alternative genomic backbone.

Upon selection and plaque purification of the phages, lysogens were generated in order to determine how the incorporation of a phage altered the bacterial host cell's phenotype. It was proposed that prophage incorporation would have an

effect on bacterial growth rates and antibiotic resistance due to the increased genetic size of the bacterium upon phage integration. Both Lyon and colleagues and Clowes and colleagues described the ability of plasmids to confer antimicrobial resistance to *S. aureus* through conjugation (Clowes, 1972, Lyon and Skurray, 1987). In 1975, Lacey described that the bacteriophages of *S. aureus* may be aiding antimicrobial resistance within the bacterial isolate (Lacey, 1975). These studies support these data presented here. Bacteriophage encoded antimicrobial resistance has been recently reviewed by Davies *et al*. (2016).

Bacterial growth rates were seen to be elevated in 75 % of the lysogens compared to the naïve PAO1. These data also support previous findings that *E. coli* strains harbouring a prophage region exhibit elevated growth rates in comparison to naïve *E. coli* strains (Edlin *et al*., 1977, Edlin *et al*., 1975, Lin *et al*., 1977).

The lysogen with the phage isolated from the < 10 BR patient displayed a slower growth rate over an 18 hour period whilst the other lysogens showed increased growth rates upon phage integration. These is proposed due to the idea that < 10 BR P. aeruginosa isolates and their associated phages have had the shortest amount of time to co-evolve alongside their bacterial host and so are not as productive/infective as phages isolated from adult CF patients. However, disease time is not correlated to the length of time that the patient has been colonised with *P. aeruginosa* with but it is still possible to make hypothesis as long as this caveat is also considered.

This trend compares to the original observations in chapter 3 that the phages from the most naïve environments, < 10 BR and paediatric CF, were the least infective in comparison to the phages isolated from > 10 BR and adult CF. These

data would indicate that the extra genetic burden on the host cell is not expensive for the bacterium.

This elevation in growth would also lead to the assumption that phages are driving an increase in metabolism within their hosts which drives more rapid proliferation within a bacterial culture. These metabolites may then be released into the environment upon the host cell's death providing a more nutrient rich environment for surrounding cells and thus, promoting an elevated growth rate in these surrounding cells. Prophage incorporation into these clinical *P. aeruginosa* isolates is therefore, showing a positive selective advantage in terms of increasing growth rates and possibly allowing *P. aeruginosa* to become the predominant bacterial species.

Antibiotic tolerance is elevated in the lysogens indicating that the phages are subverting the bacterial cell and offering an evolutionary advantage. This potentially, is maintaining both the phage and the bacterial cell in an environment which is constantly under assault with anti-pseudomonal antibiotics. Even when treating the lysogens with clinically relevant antibiotics, it is determined that the phage increases the bacterial cell's tolerance towards clinical anti-pseudomonal antibiotics. It is also possible to consider the effect that phage incorporation has on clinical treatments as sub-optimal treatment levels of antibiotic may actually induce phage induction (Wright *et al.*, 2013). This phage induction would enable the phages to spread around a bacterial population and infect other host cells, so conferring this tolerance to other receptive bacterial cells.

In order to be critical about the antibiotic tolerance profiles described previously, cell growth was normalised in relation to growth of the bacteria without the pressures of antibiotic treatment. The smallest change in growth in the presence of the drug was seen in the CF *P. aeruginosa* isolates and this may indicate that

these phages alone are driving the alterations seen in the tolerance profiles. It could be premised that the CF phages are capable of this due to their longevity within the chronic lung environment, which may have allowed the phage to accrue the functions required to increase their bacterial host cell's resistance. The notion that the CF phages are the most evolved in the chronic lung supports data from chapter 3, which indicates that longevity and colonisation within the chronic lung alters as the patient ages. The largest change in growth in the presence of an antimicrobial was seen when studying the BR phages. BR phages may need to subvert their bacterial host cells rapidly as they are new colonisers to the chronic lung environment and so therefore, have not accrued the functions required to alter tolerance independently, like the CF phages.

The results generated for the antibiotic tolerance profiles were based upon an increase in absorbance. In order to determine that the increases in the absorbance values were phage derived and due to cellular growth rather than a build-up of cellular debris, it was decided to undertake cell counts. It was also pertinent to look at the free phage titre to see if phages were propagating under the pressure of the antimicrobial. Colistin did not show any plaques when plaque assays were undertaken, potentially indicating that lysogenic events were occurring throughout the experiment resulting in no detectable free phage after 9 hours of growth. Again this shows that prophage inclusion is conferring a positive selective advantage to the bacterial isolates in this investigation, as their ability to withstand antimicrobial pressures is elevated in comparison to non-lysogenic strains.

This chapter also studied the interaction between phages and their host's pili, as pili have previously been described as a key site for phage adsorption and infection (Ochman *et al.*, 2000, Inouye *et al.*, 1991, McShan *et al.*, 1997). Mahenthiralingam *et al* (1994) proposed that adult CF isolates showed a

reduction in twitching compared to paediatric CF isolates, so initially twitching motility assays were undertaken to support these findings. These data indicated that the median levels of twitching motility were the same for the adult CF and paediatric CF isolates. However, the distribution of the motility showed that the paediatric CF isolates were less motile compared to the adult CF isolates, which is in contrast to previously published data (Mahenthiralingam *et al.*, 1994, Kus *et al.*, 2004). It is however, pertinent to mention that the paediatric CF cohort sample size was only n = 10. It is interesting to note that the BR *P. aeruginosa* isolates could twitch more than the CF *P. aeruginosa* isolates, supporting other published data which shows that the ability to twitch is lost during long term colonisation in CF (Mahenthiralingam *et al.*, 1994).

It was also demonstrate in this chapter that the addition of exogenous phage altered the motility of the bacterial isolates, as phage integration may provide both a positive and negative advantage for the bacterium. Twitching motility assays showed that the median values were greater for the *P. aeruginosa* isolates without the addition of exogenous self-induced phage. These data could indicate that in most instances phage may in fact be impeding the motility of a bacterial isolate. However, 14 *P. aeruginosa* isolates showed an elevation in twitching upon the addition of exogenous self-induced phage but only 8 of these isolates could be classed as motile when utilising the LESB58 cut-off value. *P. aeruginosa* strain type did not show any trends in these 14 isolates as they were all classed as unique upon VNTR typing. So these phages maybe conferring a positive advantage to their hosts as they are increasing the distance that the bacterium can move so therefore, increasing bacterial survival rates through the ability of the bacterium to reach more nutrients.

Genes encoding for type IV pili were detectable via PCR in over 70 % of the *P. aeruginosa* isolates involved in this investigation, enforcing the essential role that

type IV pili may have in terms of motility. Multiple PCR products were detected for some *P. aeruginosa* isolates but this may be due to *P. aeruginosa* cells often harbouring multiple type IV pili but only one flagellum (Skerker and Berg, 2001). The increase in metabolism proposed when studying the growth profiles is also pertinent here, as the increase in freely available metabolites could also increase the amount of energy that the bacterial isolates have to utilise on motility. As previously stated, these metabolites may be released into the surrounding cellular environment upon host cell lysis thus, providing more metabolites for the bacteria to translocate towards.

All these studies have shown that prophage formation provides a selective advantage for the bacterial host in terms of growth, antimicrobial resistance and twitching.

5. METABOLITE AND METABOLISM CHARACTERISATION OF *PSEUDOMONAS AERUGINOSA* PELLICLE FORMATION AND THE IMPACT OF CONVERTING PHAGES

5.1. INTRODUCTION

Chapters 3 and 4 have shown that *Pseudomonas aeruginosa* (*P. aeruginosa*) phages from chronic respiratory diseases carry genes that can be additive in nature and subvert host cellular function. Chapter 3 focused on the mixed temperate phage communities induced from cystic fibrosis (CF) and bronchiectasis (BR) *P. aeruginosa* isolates. The chapter indicated that the adult CF phage communities were the most infective and this was proposed to be a result of their longevity within the chronic lung. In order to confer more specific functions to the temperate bacteriophages of *P. aeruginosa*, 4 phages (one from each clinical aetiological subgroup) were plaque-purified and stable lysogens were created in a PAO1 background. In chapter 4, these lysogens showed advantages compared to the non-lysogenic PAO1 in terms of antibiotic tolerance and increased growth rate. In order to determine the added functionality these phages encode, this chapter focuses on studying shifts in metabolism and metabolite production that may arise due to the infection of a temperate phage and their dissemination across a bacterial community.

A key focus was to determine how these temperate phages subvert cellular function and how they aid bacterial growth, adaptation and evolution. This may be through the utilisation of new processes or differential expression of native biological pathways by the infecting phages. Pellicle cultures were generated for these studies as we propose that they may be more representative of the chronic lung. They form at the liquid-air interface which in the chronic lung would be

between the mucus layer and the air in the lower lung (Friedman and Kolter, 2004b). The use of pellicle cultures may provide us with a more representative model of the relationships occurring between phages and their *P. aeruginosa* isolates *in situ*.

Phages carry genetic material that when integrated into their bacterial host cell can influence virulence and/or the metabolic activity of their host (Nanda *et al.*, 2015, Brown *et al.*, 2006, Fortier and Sekulovic, 2013). For this reason, the effect of prophages generated in this thesis wanted to be studied through various different methodologies to determine the impact of prophage formation on bacterial metabolism.

5.1.1. Biofilms and their environmental importance

The colonisation pattern and pathogenicity of *P. aeruginosa* in the CF lung is multifactorial and there are numerous methods that the bacterium and its associated phage can utilise in order to survive. *P. aeruginosa* can subvert host defences due to its ability to form biofilms; these are bacterial cell aggregates which form when an extracellular matrix (ECM) is generated and surrounds the bacterial cells. This matrix protects the internalised cells from external stimuli, such as antimicrobials and antibiotics (Drenkard and Ausubel, 2002a). There are some general principles that are required for biofilm formation and these are; a surface covered in nutrients for the initial colonisation, a metabolically active bacterium for surface attachment and adherence, an adequate nutrient supply to promote cellular replication and the associated cell growth along with ECM generation (Costerton *et al.*, 1995, Costerton, 1995). It is believed that biofilm formation follows a strict and order pathway that is due to the up regulation and down regulation of certain genes within the bacterial genome.

A key gene that is often responsible for biofilm formation is *rpoS* which encodes for the protein RpoS, a subunit of RNA polymerase. RpoS is involved in the expression of many genes that may have roles in the stationary growth phase of *P. aeruginosa*, whilst in *E. coli* it is believed that this protein is one of the main regulators in the bacterium's stress response system (Hengge-Aronis, 2002). Schembri *et al* (2003) described that 46 % of the genes differently up regulated in a DNA microarray of an *E. coli* biofilm were under the control of RpoS, so indicating the key role that this protein plays during biofilm formation in the stationary phase growth (Schembri *et al*., 2003). It is believed that the transition of a Gram negative bacteria from exponential growth into the stationary phase is under the control of RpoS (Latifi *et al*., 1996). It has been determined that the cellular level of *E. coli* RpoS increases dramatically during the late stages of logarithmic growth and as the bacterium enters into stationary phase (Hengge-Aronis, 1993, Lange and Hengge-Aronis, 1994). A homologue of RpoS in *P. aeruginosa* follows the same pattern (Kan and Hideo, 1994). *P. aeruginosa* also harbours a multifactorial and hierarchical Quorum Sensing (QS) cascade which is involved in the activation of RpoS during stationary phase growth (Latifi *et al*., 1996).

The development of biofilms in the lower lung is perceived to be one of the many reasons which supports the establishment of *P. aeruginosa* as the dominant bacteria. It is believed that some bacterial communities commonly reside in biofilms in nature, even if in the laboratory they form planktonic communities (Costerton *et al*., 1995).

Biofilm formation upon medical implants related to cardiac disease is a serious health problem worldwide; cardiac disease in 2006 was one of the major causes of morbidity in the USA (Padera, 2006). Treatment of biofilms on medical implants usually involves the removal of the contaminated item in order to try and

reduce the bacterial load present thus, containing the spread of biofilm forming bacterial species (Gjersing *et al.*, 2007). Staphylococcal infections have been shown to have a fold increase in antibiotic resistance of between 10 – 1000 when the bacterium is growing within a biofilm and encased in the ECM (Campoccia *et al.*, 2005, Nishimura *et al.*, 2006, LaPlante and Woodmansee, 2009).

5.1.2. Pellicle formation

P. aeruginosa has been previously shown to form different biofilm structures depending on the environmental niche that the bacterium inhabits (Friedman and Kolter, 2004a). A pellicle is a biofilm that assembles at the air-liquid interface in liquid cultures (Friedman and Kolter, 2004b) and the *pel* locus is believed to be essential (amongst other genes) for the formation of biofilms and pellicles in PA14 (Friedman and Kolter, 2004a). Friedman and Kolter (2004a) when studying *P. aeruginosa* showed that the genes of the *pel* locus are responsible for the formation of a glucose-rich matrix and this matrix is essential for the stability and longevity of the biofilm. The *pel* locus is composed of an operon of 7 adjacent genes which span 12.2 kb of the PAO1 genome. This operon is also found in other *P. aeruginosa* isolates as there is a region with 98 % similarity in the PA14 genome (Friedman and Kolter, 2004a).

Homologues to this gene cluster are found in multiple other diverse bacteria, such as *Ralstonia solanacearum* (aerobic Gram negative plant pathogen), *Ralstonia metallidurans* (non-sporing Gram negative bacterium), *Geobacter metallireducens* (Gram negative metal reducing *proteobacterium*) and *Burkholderia fungorum* (*proteobacterium*) (Friedman and Kolter, 2004a). Elevated expression of *pel* in small colony variants (SCVs) of *P. aeruginosa* isolated from CF patients is proposed to aid *P. aeruginosa* colonisation and persistence within the lower lung (Starkey *et al.*, 2009). These data may show

how essential this locus is for *P. aeruginosa* survival. *Pel* is required for PA14 pellicle maturation but it is not essential for pellicle initiation, so it is believed that *pel* is involved in the later stages of pellicle generation (Friedman and Kolter, 2004a).

The *psl* locus is also involved in polysaccharide synthesis within the pellicle. Psl is known to aggregate *P. aeruginosa* to the airway epithelial cells through mucin interactions, so ensuring the maintenance of *P. aeruginosa* in the lower lung environment (Friedman and Kolter, 2004a, Friedman and Kolter, 2004b, Jackson *et al.*, 2004, Vasseur *et al.*, 2005, Ma *et al.*, 2009). The 11 predicted protein products of *psl* are all homologues of carbohydrate processing proteins and it has been determined that the *psl* locus is involved in the generation of a mannose-rich matrix in *P. aeruginosa* (Friedman and Kolter, 2004b). The *psl* locus is conserved between PAO1 (Stover *et al.*, 2000) and *Pseudomonas syringae* DC300 (Buell *et al.*, 2003). In 2013, it was proposed that the gene products of the *psl* locus are of equal importance in biofilm formation as the gene products of the *pel* locus (Huse *et al.*, 2013). *Psl* is involved alongside *pel* in pellicle stability but the gene products of *psl* alone are sufficient for pellicle maturation to occur (Friedman and Kolter, 2004b). This observation leads to the hypothesis that *psl* is involved throughout the pellicle production process.

5.1.3. Quorum Sensing in *P. aeruginosa*

P. aeruginosa has a hierarchical system of two QS systems; LasI/LasR (Passador *et al.*, 1993) and RhlI/RhlR (Brint and Ohman, 1995). Both LasI and RhlI are the autoinducer synthases that catalyse the formation of the autoinducers; *N*-(3-oxododecanoyl)-L-homoserine lactone (Pearson *et al.*, 1994) and *N*-(butyryl)-homoserine lactone, respectively (Pearson *et al.*, 1995). When a

high cellular density of *P. aeruginosa* cells occurs, LasR binds to its autoinducer (*lasI* product) which leads to the activation of genes which are involved in virulence factor production (Davies *et al.*, 1998, De Kievit and Iglewski, 2000, Jones *et al.*, 1993, Passador *et al.*, 1993, Miller and Bassler, 2001). The LasR-autoinducer complex can also activate the second QS system of *P. aeruginosa*; RhlI/RhlR (Ochsner and Reiser, 1995). Upon expression of *rhlR* through the activation of the LasR-autoinducer complex, RhlR can bind to its autoinducer molecule (product of *RhlI*). The RhlR-autoinducer complex can then activate its own set of virulence genes which are involved in a range of processes within the *P. aeruginosa* host including alkaline proteases (Gambello *et al.*, 1993, Latifi *et al.*, 1996), rhamnolipid bio-surfactants (Ochsner *et al.*, 1994, Latifi *et al.*, 1996), exotoxin A (Blackwood *et al.*, 1983, Brint and Ohman, 1995) and elastase (Blackwood *et al.*, 1983, Brint and Ohman, 1995). Figure 5.1 shows the pathways that are involved in QS in *P. aeruginosa* (Dubern and Diggle, 2008).

In order to control QS in *P. aeruginosa*, the LasR-dependent autoinducer prevents the RhlI autoinducer binding to its own receptor; RhlR (Pesci *et al.*, 1997). It is assumed that this second level of control of RhlI/RhlR by LasI/LasR is to ensure that the systems function in a sequential manner. Aside from these two aforementioned QS systems, there is an additional autoinducer; 2-heptyl-3-hydroxy-4-quinolone (PQS) (Pesci *et al.*, 1999). Expression of this autoinducer requires LasR and in turn, this molecule activates the expression of the autoinducer molecule of RhlI. It is believed that again this molecule adds another level of control to ensure that QS occurs sequentially and in an ordered fashion in *P. aeruginosa*.

QS mutants have been described as a common phenotypic feature in chronic *P. aeruginosa* populations colonising the CF lung. These mutants induce host inflammatory responses in respiratory epithelial cells generating an elevation in

proinflammatory cytokines and neutrophil recruitment. The influx of these inflammatory metabolites increases tissue damage within the CF lung and this leads to a rapid decline in patient health (Winstanley and Fothergill, 2009, Heurlier *et al.*, 2005, LaFayette *et al.*, 2015, Mowat *et al.*, 2011).

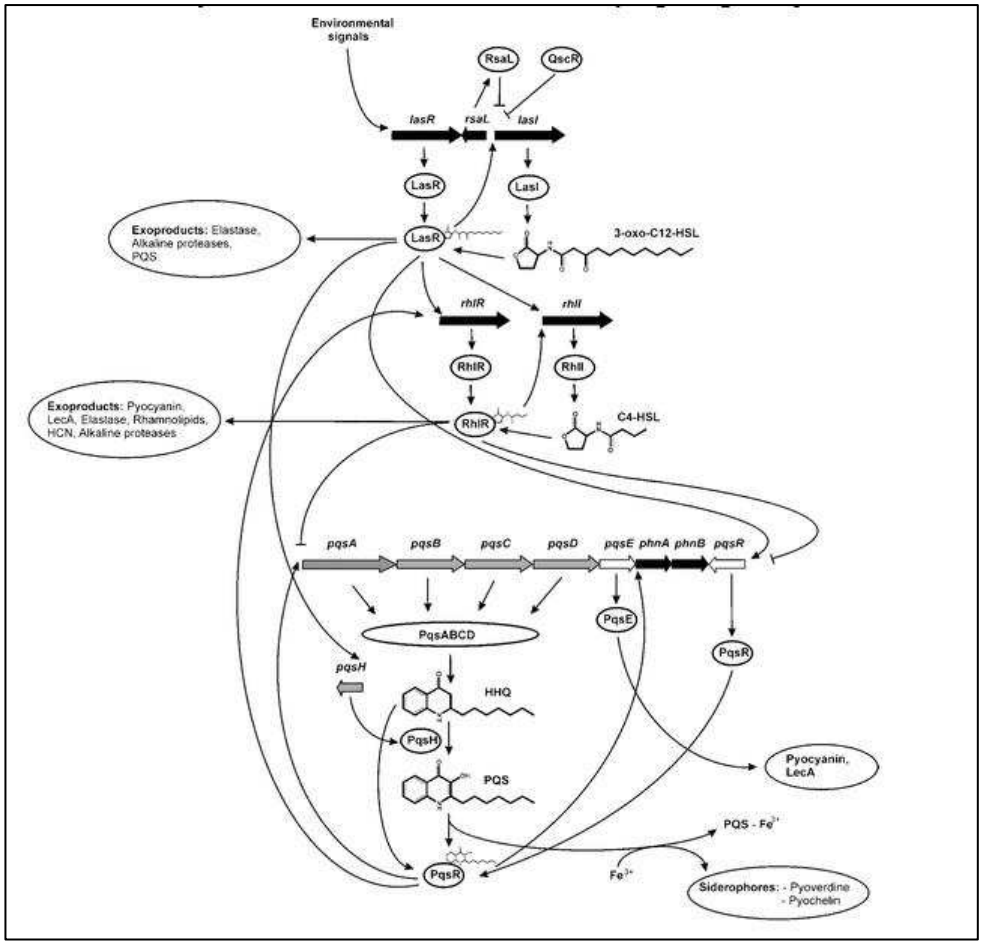


Figure 5.1: *P. aeruginosa* cell-to-cell signalling resulting in the formation of biofilms (Dubern and Diggle, 2008). The two QS systems are interlinked (RhlR/RhlI) by the LasR system. The various endpoints are shown on the figure including pyocyanin, elastase, 2-heptyl-3-hydroxy-4-quinolone and alkaline protease to name a few. The level of complexity is shown in this figure and it emphasises that the QS system is multifactorial.

5.1.4. Metabolomics

Metabolomic studies using high-resolution liquid chromatography combined with mass spectrometry offer an approach to identify and quantify the metabolite profile of a particular biological sample or organism. These products are normally either the end point of cellular regulatory processes, intermediates of pathways, or other signalling molecules (Reo, 2002, Fiehn, 2002, Zhang and Powers, 2012). Metabolomics links phenotypic and genotypic studies in order to generate a more comprehensive overview of the functionality of a particular biological community (Weckwerth, 2003, Weckwerth and Morgenthal, 2005, Fiehn, 2002). The technology is rapid and discrete enough to detect the steady state concentrations of the metabolites, and then determine any fluctuations that may occur upon the alteration of an external stimuli (Weckwerth and Morgenthal, 2005). Metabolomic analysis doesn't require the complete isolation and characterisation of individual metabolites within a biological sample instead it captures a snapshot or 'fingerprint' of the status of the metabolome (Fiehn, 2002, Ellis *et al.*, 2007). Untargeted metabolomic systems offer semi-quantitative information about the possible metabolites present and their abundance in a biological system (Chokkathukalam *et al.*, 2014). Mass spectroscopy (MS) and nuclear magnetic resonance (NMR) are the primary analytical tools used by researchers when undertaking metabolomic studies and these techniques are often preceded by a liquid chromatography (LC) stage (Zhang and Powers, 2012).

5.1.5. Liquid chromatography mass spectroscopy

Liquid chromatography mass spectroscopy (LCMS) is one of the most commonly used analytical techniques (Snyder *et al.*, 2011), and is often used for the quantification of unknown metabolites (Josephs and Sanders, 2004, Shockcor *et*

al., 2003, Yang *et al*., 2002, Weckwerth and Morgenthal, 2005). LCMS is composed of an LC stage which can separate samples according to molecular weight and an MS stage which ionises the compounds within a sample in order to generate the highest definition of metabolites.

The principle of chromatography is based upon the physical separation of a sample with the addition of a solvent (Niessen, 2006, Covey *et al*., 1986, Arpino *et al*., 1974, Arpino *et al*., 1979). LC functions via two phases a mobile and a stationary phase; the mobile stage is the solvent and the stationary phase is the matrix of the column. The column is commonly around 10 – 30 cm in length and has an internal diameter of around 3 – 4.6 mm (Niessen, 2006). The column is tightly and uniformly packed with silica beads which have a diameter of around 3 – 5 µM. These beads are usually porous in order to increase the surface area that is available for molecule binding. Two solvents are pumped across the column; one (solvent A), by convention is an aqueous solvent such as water with an addition of 0.1 % formic acid and the second, (solvent B) by convention is an organic solvent such as acetonitrile, methanol or propanol, again with the addition of 0.1 % formic acid. The addition of formic acid to both solvents improves chromatographic peaks along with providing a source of protons which are invaluable in the ionisation step of LCMS (Niessen, 2006).

5.1.5.1. Mass spectroscopy

MS is an analytical technique that can provide both quantative (molecular mass) and qualitative (structure) results for an individual sample upon its ionisation (Mann and Fenn, 1992, Mann *et al*., 1989). MS is the preferred platform for metabolite identification due to its high sensitivity and vast metabolite detection capabilities but it is also a destructive process due to the ionisation step

(Theodoridis *et al.*, 2012, Chokkathukalam *et al.*, 2014, Zhang and Powers, 2012). MS is composed of 5 steps with the initial step being the introduction of the separated LC sample to the ionisation source. Electrospray ionisation is used to convert the liquid LC samples into ions for the MS stage. This process involves the bombardment of the samples with highly powered electrons followed by sample evaporation allowing for the generation of both positive and negative ions (Niessen, 2006, Ho *et al.*, 2003, Mann and Fenn, 1992). The newly charged ions become gaseous due to electric field alterations. These gaseous ions are then accelerated through the system which allows for subsequent analysis to be undertaken (Ho *et al.*, 2003). Further targeted MS studies are needed to confirm the true identify of an MS sample, usually using a fragmentation stage or comparison to standard compounds (Dunn *et al.*, 2013, Chokkathukalam *et al.*, 2014) but untargeted studies such as this one allow for a representation of the metabolome of a sample to be elucidated.

5.1.6. Metabolomics and biofilms

Gjersing *et al* (2007) showed that the overall metabolism of *P. aeruginosa* was similar between planktonic and biofilm cells when comparing the extracellular metabolites present in spent media generated by continuous batch cultures via NMR. This indicates that the extracellular metabolites generated by *P. aeruginosa* cells are similar regardless of the growth stage whereas the intracellular metabolites were shown to vary when studying planktonic/biofilm cellular communities. Lower spectral peak intensities were determined for biofilm cells compared to planktonic cells when looking at intracellular metabolites potentially, indicating that different pathways are being utilised in the different growth stages (Gjersing *et al.*, 2007). Sonkar *et al* (2012) investigated the effect that the filamentous phage, Pf1, had on the metabolites detected in *P.*

aeruginosa planktonic cultures. Prior to phage infection at mid exponential growth, the cultures had similar metabolite profiles but upon phage infection the metabolites varied dramatically with the phage infected culture showing a propensity for low molecular weight metabolites. Pf1 infected cultures had a high occurrence of branched chain amino acids, lactate, acetate, pyruvate, glyceraldehyde, indole, and ascorbate.

The most actively expressed genes in *P. aeruginosa* biofilms are proposed to have a bacteriophage origin; Pf1 (Hill *et al.*, 1991, Whiteley *et al.*, 2001). Pf1 transcripts have also been shown to be nearly 100 – 1000 fold greater in continuous biofilm reactor cultures compared to planktonic culture reactor communities (Whiteley *et al.*, 2001). The observation of more energy metabolites in Pf1 infected cultures may indicate that these cultures are more active and so the phage is providing the bacterial culture with a metabolic advantage. The observation of acetate and lactate is consistent with previous findings (Schleheck *et al.*, 2009). Pf1 infected cultures also exhibited elevated levels of agmatine; a polyamine utilised by *P. aeruginosa* to form biofilms (Sonkar *et al.*, 2012).

5.1.7. Metabolism studies and quantifying biofilms

Metabolism studies targeting a marker of respiration are often used in conjugation with other methods such as metabolomics, in order to increase our knowledge of a particular biological system. Studies on bacterial biofilms using metabolism marker assays can confer putative information relating to the activity of the biofilm. Colorimetric assays can be used to determine cell/biofilm viability (Peeters *et al.*, 2008). They discriminate between viable and dead cells in a biofilm community as a colourless substrate is utilised which can be modified to a coloured compound by an actively respiring cell (Mosmann, 1983). Two stains

are commonly used in these metabolic assays, these are 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2,3-bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) (Peeters *et al.*, 2008, Gabrielson *et al.*, 2002, McCluskey *et al.*, 2005).

5.1.7.1. XTT assays

Finding assays which are linked to core energy metabolism offers a way to determine changes in cell respiration, especially when the bacterium is grown under different environmental conditions. There have however, been observations of poor correlation between XTT results and cell counts. It is believed that this alteration is due to microbial metabolic activity not always being solely affected by cell viability (Hatzinger *et al.*, 2003, Honraet *et al.*, 2005, Simões *et al.*, 2007). It is also possible that other cellular factors might cause the reduction of the XTT dye thus, introducing potential bias and erroneous results when quantifying the number of viable cells within a biofilm (Bedwell *et al.*, 2001, McCluskey *et al.*, 2005, Al-Bakri and Afifi, 2007). Erroneous results can also be generated when comparing inter- and intra-species differences especially in the *Candida albicans* species and when investigating yeast at different stages of growth (Kuhn *et al.*, 2003, Honraet *et al.*, 2005, Al-Bakri and Afifi, 2007, Kuhn *et al.*, 2002a, Kuhn *et al.*, 2002b). However, the reasoning behind this alteration in XTT results between various bacterial species is currently not understood.

XTT assays are based upon the reduction of the XTT tetrazolium salt to the water soluble dye, formazan, by metabolically active cells. This reduction is triggered by succinyl-dehydrogenase enzymes which are present in the electron transport system of the bacteria (McCluskey *et al.*, 2005, Al-Bakri and Afifi, 2007). Formazan can be detected in cell supernatants via spectrophotometry

because the colour changes from clear to orange. An increase in cellular respiration directly correlates to an increase in dye detection through the cleavage of XTT (Roehm *et al.*, 1991, Peeters *et al.*, 2008, Kuhn *et al.*, 2002b). XTT assays have also been used to determine the minimum inhibitory concentration of phage required in phage therapy experiments (Vipra *et al.*, 2013). The XTT assay was used in this instance as a rapid alternative to traditional cell culture methods as it was possible to undertake the assay on both lytic and temperate phages (Vipra *et al.*, 2013).

Metabolomic and metabolism studies were utilised in this chapter to determine how the inclusion of a temperate phage into a pellicle culture alters the functionality of the pellicle and how the culture develops. As stated previously, our main focus was to determine how phages alter bacterial growth, adaptation and evolution and it was considered that these techniques may give us a good understanding of the underlying processes which may be manipulated upon phage incorporation into a pellicle.

5.2. AIMS

The aim of this investigation was to determine through metabolomics and metabolism studies, how phages influence the progression of pellicle development in *P. aeruginosa*. This was achieved by observing alterations in the metabolites produced and/or changes in metabolism during pellicle growth. In order to allow for more detailed analysis to be undertaken, these studies focused on a smaller cohort of *P. aeruginosa* hosts from each clinical aetiology (n = 4).

The metabolism study using XTT involved *P. aeruginosa* hosts from each of the clinical aetiologies (n = 4) and a small scale cross infection involving the mixed

phage communities induced from each of the clinical *P. aeruginosa* isolates. This was undertaken to determine whether the host was most adapted to its own phage thus, showing an elevation in metabolism or whether the host metabolic rate was influenced solely by the incoming phage.

The metabolomic study in this chapter utilised the lysogens created in chapter 4, which used PAO1 as the bacterial host cell. Bacteriophages can subvert their bacterial host cell therefore, we hypothesised that during pellicle formation the metabolite profile would alter and the metabolites detected via LCMS may be different between the naïve, uninfected cultures when compared to the phage infected cultures.

5.3. RESULTS

5.3.1. Pellicle formation

The method shown in section 2.4.2 was the final method chosen for this process but preliminary work was undertaken in order to generate the most efficient process. The phages induced and utilised in chapter 4 were also used.

Preliminary study: The mixed phage communities used in the preliminary study were CF47 (Paediatric CF), CF57 (Adult CF), BR152 (< 10 BR) and BR153 (> 10 BR). This work also focused upon determining the best time for the addition of a phage into a growing pellicle culture. The times tested were 0, 24 hours post pellicle formation, 36 hours post pellicle formation and 48 hours post pellicle formation. The observations are all detailed in tables 5.1 – 5.4 and images of the pellicles during growth and after harvesting are shown in figure 5.2.

The results shown in these tables are based on visual observations taken every 12 hours for a 108 hour period. The initial observation was taken 24 hours post culture set-up as it was perceived that this time point would allow for the pellicle to have started developing. Large variations were seen in the results but these variations were potentially generated due to the cultures drying out, this lead to the altered protocol used in the final method. With the exception of the pellicle products generated for the two most naïve *P. aeruginosa* isolates (CF47 and BR152), good growth was seen throughout and no major differences were seen between the different time points nor the different phage addition time points. The exceptions seen for the CF47 and the BR152 pellicles can be attributed towards the pellicle cultures drying out throughout the incubation process. The pellicle for CF47 did however, have the poorest pellicle growth when visual observations were undertaken but this is possibly due to the naivety of this *P. aeruginosa* isolate and therefore, the ability to form robust biofilms may not be as evolved in this isolate compared to the other *P. aeruginosa* isolates in this cohort. Naivety needs to be concluded with caution as just because the CF patient is only a paediatric, doesn't mean that the patient has been only been colonised with *P. aeruginosa* for a short time period.

For further study, it was decided to add the phage lysates directly to the culture (t = 0). This was because no differences were seen in the preliminary study between the various time points. It was also decided to grow the pellicles for 102 hours but extract replicate samples after 36, 72 and 102 hours of growth in order to generate results which were the most representative of the pellicle formation process. This would show any potential metabolomic and respiration alterations that may be occurring throughout the process.

Table 5.1: Preliminary work into pellicle formation in paediatric CF isolates. Paediatric CF *P. aeruginosa* host and the addition of phages induced from the same *P. aeruginosa* host showing variations in the biofilm formation process. ++++ Good growth, +++ Thin growth, ++ Starting growth observed, + very small pellicle observed, - no observable pellicle or very little and – dried out. These results were all achieved through visual observations of the cultures.

Hours	**CF47 MINUS ϕ**	**CF47 PLUS ϕ (straight away)**	**CF47 PLUS ϕ (24 hours)**	**CF47 PLUS ϕ (36 hours)**	**CF47 PLUS ϕ (48 hours)**
24	-	++	++	++	++
36	+++	+++	+++	+++	+++
48	+++	+++	+++	+++	+++
60	-	-	+	+	+
72	-	-	+	+	+
84	-	-	+	+	+
96	-	-	+	+	+
108	-	-	+	+	+

Table 5.2: Preliminary work into pellicle formation in adult CF isolates. Adult CF *P. aeruginosa* host and the addition of phages induced from the same *P. aeruginosa* host showing variations in the biofilm formation process. ++++ Good growth, +++ Thin growth, ++ Starting growth observed, + very small pellicle observed, - no observable pellicle or very little and – dried out. These results were all achieved through visual observations of the cultures.

Hours	**CF57 MINUS ϕ**	**CF57 PLUS ϕ (straight away)**	**CF57 PLUS ϕ (24 hours)**	**CF57 PLUS ϕ (36 hours)**	**CF57 PLUS ϕ (48 hours)**
24	-	++	++	++	++
36	+	++	++	++	++
48	++++	++++	++++	++	++
60	++++	++++	++++	++++	++++
72	++++	++++	++++	++++	++++
84	++++	++++	++++	++++	++++
96	++++	++++	++++	++++	++++
108	++++	++++	++++	++++	++++

Table 5.3: Preliminary work into pellicle formation in < 10 BR isolates. <10 BR *P. aeruginosa* host and the addition of phages induced from the same *P. aeruginosa* host showing variations in the biofilm formation process. ++++ Good growth, +++ Thin growth, ++ Starting growth observed, + very small pellicle observed, - no observable pellicle or very little and – dried out. These results were all achieved through visual observations of the cultures.

Hours	BR152 MINUS ϕ	BR152 PLUS ϕ (straight away)	BR152 PLUS ϕ (24 hours)	BR152 PLUS ϕ (36 hours)	BR152 PLUS ϕ (48 hours)
24	++	+	++	++	++
36	++++	++++	++	++	++
48	++++	++++	--	++	++
60	++++	++++	--	--	++++
72	++++	++++	--	--	++++
84	++++	+++	--	--	++++
96	--	++++	--	--	++++
108	--	++++	--	--	++++

Table 5.4: Preliminary work into pellicle formation in > 10 BR isolates. >10 BR *P. aeruginosa* host and the addition of phages induced from the same *P. aeruginosa* host showing variations in the biofilm formation process. ++++ Good growth, +++ Thin growth, ++ Starting growth observed, + very small pellicle observed, - no observable pellicle or very little and – dried out. These results were all achieved through visual observations of the cultures.

Hours	**BR153 MINUS ϕ**	**BR153 PLUS ϕ (straight away)**	**BR153 PLUS ϕ (24 hours)**	**BR153 PLUS ϕ (36 hours)**	**BR153 PLUS ϕ (48 hours)**
24	++	+++	++	++	++
36	+++	+++	++	++	++
48	+++	+++	++	++	++
60	+++	+++	+++	++++	++++
72	+++	+++	++++	++++	++++
84	+++	+++	++++	++++	++++
96	+++	++++	+++	++++	++++
108	++++	++++	++++	++++	++++

Figure 5.2: Images to show the pellicle formation process. Image A shows the harvested pellicle whilst image B shows the pellicle whilst still residing within the 6 well cell-culture plates. The darker area in the bottom 3 wells shows a pellicle product whilst the top 3 wells represent LB control cultures.

5.3.2. Respiration variations observed in pellicles with and without addition of purified phages

As stated previously, (section 5.3.1.) the phages used for all the studies from this point forwards were the phages induced from the 4 *P. aeruginosa* strains which were selected for the generation of lysogens (chapter 4). The mixed temperate phage communities for these 4 *P. aeruginosa* isolates were utilised for the metabolism marker studies and the lysogens were used for the metabolomic investigation. The metabolism profiles of the phage infected cultures were compared to the pellicle products harvested at the same time points from naïve non-phage infected cultures. XTT raw data is shown in appendix 7.

The mixed temperate phages were used for a small scale cross-infection study in order to determine whether the metabolism marker profiles were elevated due to the addition of a self-infecting phage lysate or whether the addition of phage from a different clinical origin would drive an elevation in the metabolism profiles. It has been proposed through previous work in chapter 3, that the adult CF phages are the most infective against the entire *P. aeruginosa* cohort, so it was hypothesised here that the adult CF phages would drive the largest alteration in the metabolic profiles. This assumption can be drawn as it is proposed that these adult CF bacteriophages have a large arsenal of genes which can generate the broad infection profile and so these genes may generate large metabolic changes within their new bacterial hosts.

The metabolic potential of the pellicles was determined through the addition of 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-*2H*-Tetrazolium-5-Carboxanilide (XTT). Figure 5.3 illustrates the overall data from this study and therefore, the respiratory action of each pellicle. The negative phage control is represented by the first bar in each panel and this was the value used to determine whether there were any statistical differences between the pellicle communities.

With a few exceptions, the XTT assay results indicated that the addition of exogenous phage to a pellicle culture generated a more metabolically active pellicle. However, only a few significant observations were detected. Length of pellicle growth time did not appear to have any effect on the metabolic profiles. In general, the addition of exogenous phage 24 (adult CF) caused an elevation in the metabolic profiles of the various *P. aeruginosa* pellicle cultures however; these elevations were not always significant. Another finding that can be taken from these data is that the addition of exogenous phage 136 (> 10 BR) caused a decrease in the metabolic profiles of the various *P. aeruginosa* pellicle cultures but this trend was again not apparent in all the pellicle cultures involved and was specific when infecting certain *P. aeruginosa* backgrounds. Interestingly, the CF *P. aeruginosa* pellicles exhibited more metabolically active pellicles on average compared to the BR *P. aeruginosa* pellicles.

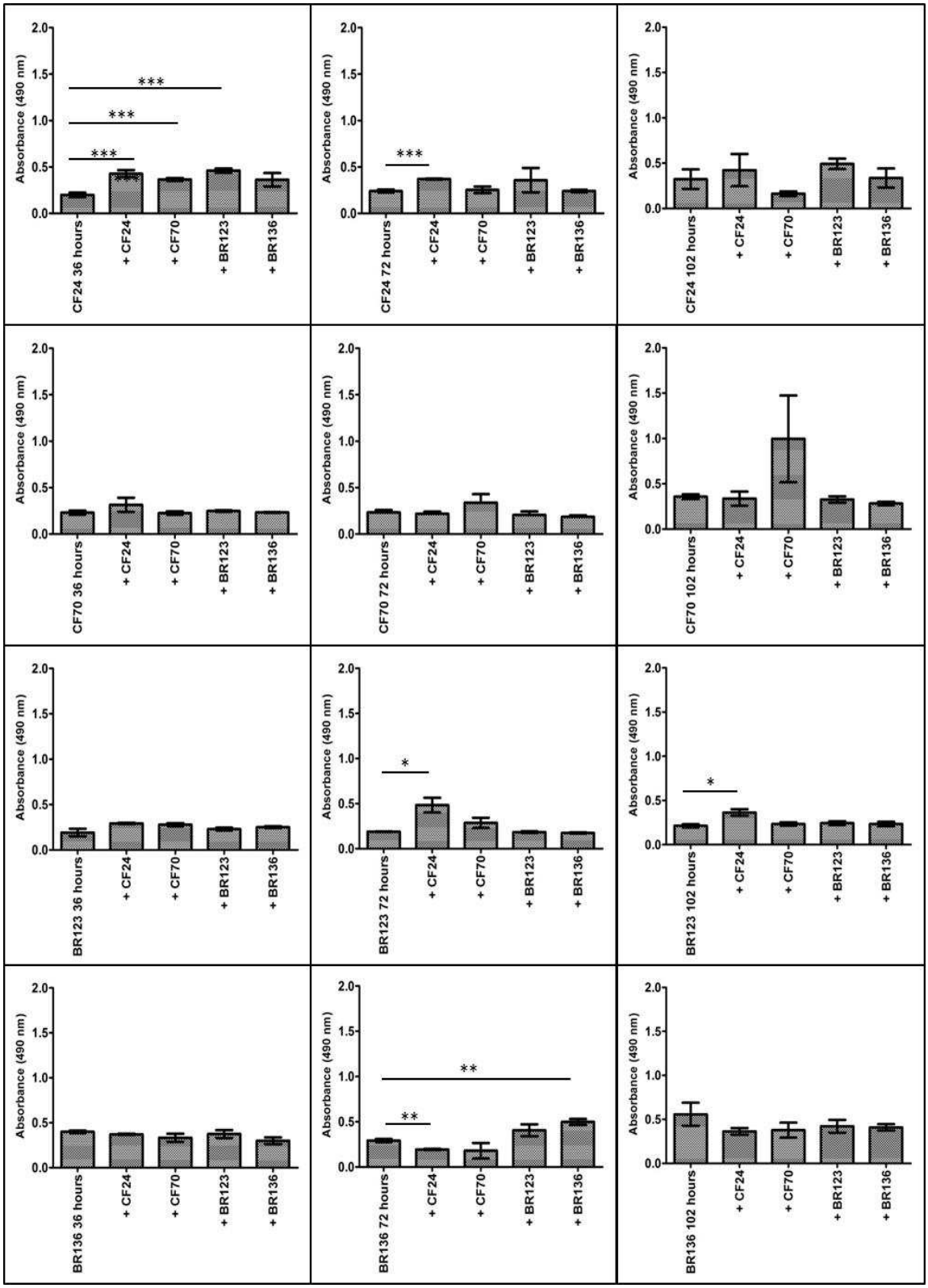

Absorbance (490 nm)
2.0
1.5
1.0
0.5
0.0
***
***
***
CF24 36 hours
+ CF24
+ CF70
+ BR123
+ BR136
***
CF24 72 hours
CF24 102 hours
CF70 36 hours
CF70 72 hours
CF70 102 hours
BR123 36 hours
*
BR123 72 hours
BR123 102 hours
BR136 36 hours
**
**
BR136 72 hours
BR136 102 hours

Figure 5.3: Absorbance readings at 490 nm, 120 minutes after the addition of the XTT to the pellicles. Non parametric t tests were utilised with two tailed p values shown in order to display the most discrete statistical significant data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001.

The naïve pellicle is represented by the first bar in each panel allowing for observations to be drawn between all the pellicles with the addition of the various mixed temperate phage lysates.

+ CF24 indicates the addition of purified phage from an adult CF *P. aeruginosa* isolate, + CF70 indicates the addition of purified phage from a paediatric CF *P. aeruginosa* isolate, + BR123 indicates the addition of purified phage from a < 10 BR *P. aeruginosa* isolate and + BR136 indicates the addition of purified phage from a > 10 BR *P. aeruginosa* isolate. The various growth periods of the pellicle are also detailed on the graph – 36 hours, 72 hours and 102 hours.

5.3.3. Orthologous PLS-DA and PLS-DA plots for 164 significant metabolites ($p \leq 0.05$ and CV ≤ 5) when the plots are separated according to the duration of pellicle growth

In order to maximise the variation in the data generated via LCMS, oPLS-DA and PLS-DA plots were generated using the discriminator of time and therefore, stage of growth, figures 5.4 and 5.5.

PLS-DA plots maximise the variation in the metabolite profiles between pellicles formed with and without phage infection whilst using duration of growth as the discriminator. The R^2Y value for the PLS-DA plot is 0.55 and the R^2Y value for the oPLS-DA plot is 0.62; values > 0.5 are thought to be representative of a robust model. Biological duplicates were performed and the samples were run on the Q-Exactive LCMS as technical triplicates.

The *E. coli* Metabolome Database (ECMDB) was used for metabolite comparison based on molecular weight. The ECMBD is based on metabolites identified in the *E. coli* strain K12, subspecies MG1655 and contains over 300 small molecule identifications with 1.4×10^2 associated enzymes and over 300 associated molecule transporters (Guo *et al.*, 2013).

After preliminary LCMS work it was decided to alter the timings utilised (data not shown for the preliminary data set). The new times were chosen in order to represent time points within the developmental process as it was proposed that the greatest alterations in the metabolite load would occur during the generation and formation of the pellicle cultures. The cultures at 36 hours post generation of the pellicle were assumed to represent biofilm cultures whereas, the previous time points could be considered more planktonic in nature. PAO1 was used as a non-lysogenic control in these data in order to allow for comparisons to be drawn between lysogenic and non-lysogenic cultures.

When looking at the individual subgroups on both the PLS-DA and oPLS-DA plots models of the 164 metabolites detected, it was seen that near identical profiles were seen for the PLS-DA and oPLS-DA plots. It is apparent from both figures 5.4 and 5.5, that the CF24 (adult CF) lysogen has the most distinct metabolite profile compared to the naïve PAO1 and this altered profile may be linked to phage infection and subversion. Most importantly, this profile is observed throughout growth. The lysogen which showed the second greatest difference compared to PAO1 was CF70 (paediatric CF). Again, this observation was found throughout the time points. In contrast, the BR123 (< 10 BR) lysogen was found to be most closely associated with the naïve PAO1 control at all the time points on both plots.

The finding that CF24 (adult CF) lysogen showed the greatest difference to the naïve PAO1 was in concordance to the results shown previously in chapters 3 and 4. In these chapters, the adult CF phages were either most infective against the entire *P. aeruginosa* cohort, showed the greatest antibiotic tolerance or showed the greatest elevation in growth compared to a non-lysogenic strain. The finding that the BR 123 (< 10 BR lysogen) shows the most similarity to the naïve PAO1 control also relates to previous findings in chapters 3 and 4, as the < 10 BR phages have been shown to be the most naïve in terms of antibiotic tolerance, infection capabilities and growth profiles.

In order to generate quantitative data to support both the oPLS-DA and PLS-DA plots described above, cell counts were undertaken for each lysogenic PAO1 strain at the various extraction points, figure 5.6. It was seen that the average cell count increased throughout the developmental process, which shows that the metabolomic profiles generated are associated to a significant increase in cellular growth.

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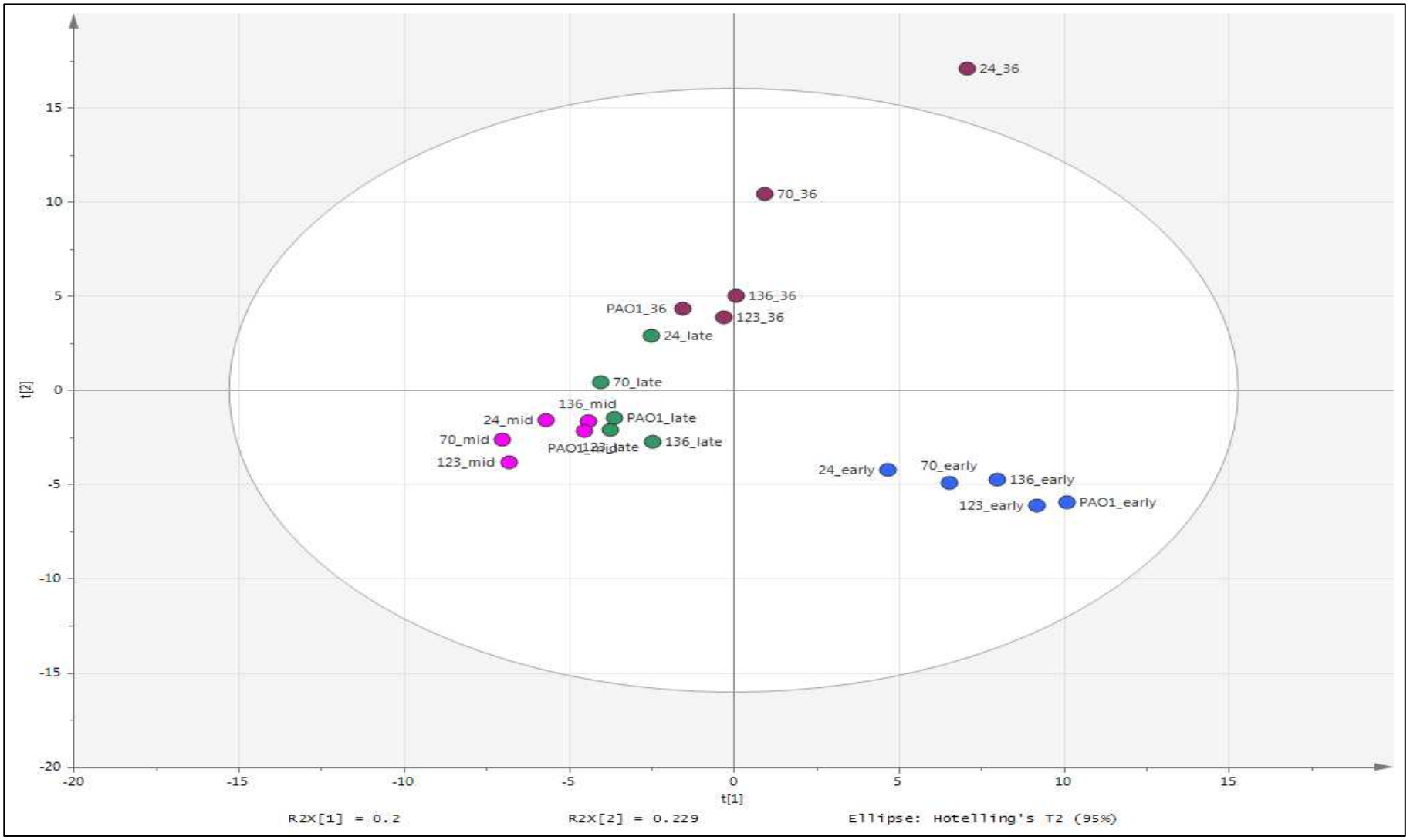
24_36
70_36
136_36
PAO1_36
123_36
24_late
70_late
136_mid
24_mid
PAO1_late
70_mid
123_late
136_late
123_mid
24_early
70_early
136_early
123_early
PAO1_early
t[2]
t[1]
R2X[1] = 0.2
R2X[2] = 0.229
Ellipse: Hotelling's T2 (95%)

Figure 5.4: A PLS-DA plot describing proposed metabolite changes for 164 significant metabolites ($p \leq 0.05$) with a CV score of ≤ 5 during pellicle formation. Lysogens were grown for ~ 3 hours, average OD_{600} 0.124 (early), ~ 5 hours, average OD_{600} 0.494 (mid) and ~ 10 hours, average OD_{600} 1.136 (late) and 36 hours to determine changes in the metabolite profile throughout the pellicle formation process.

R^2Y is 0.55 when the discriminating factor is time.

Early growth is represented by the blue data points, mid growth by the pink data points, green points represent metabolites from late growth whilst burgundy points represent the 36 hour growth profiles.

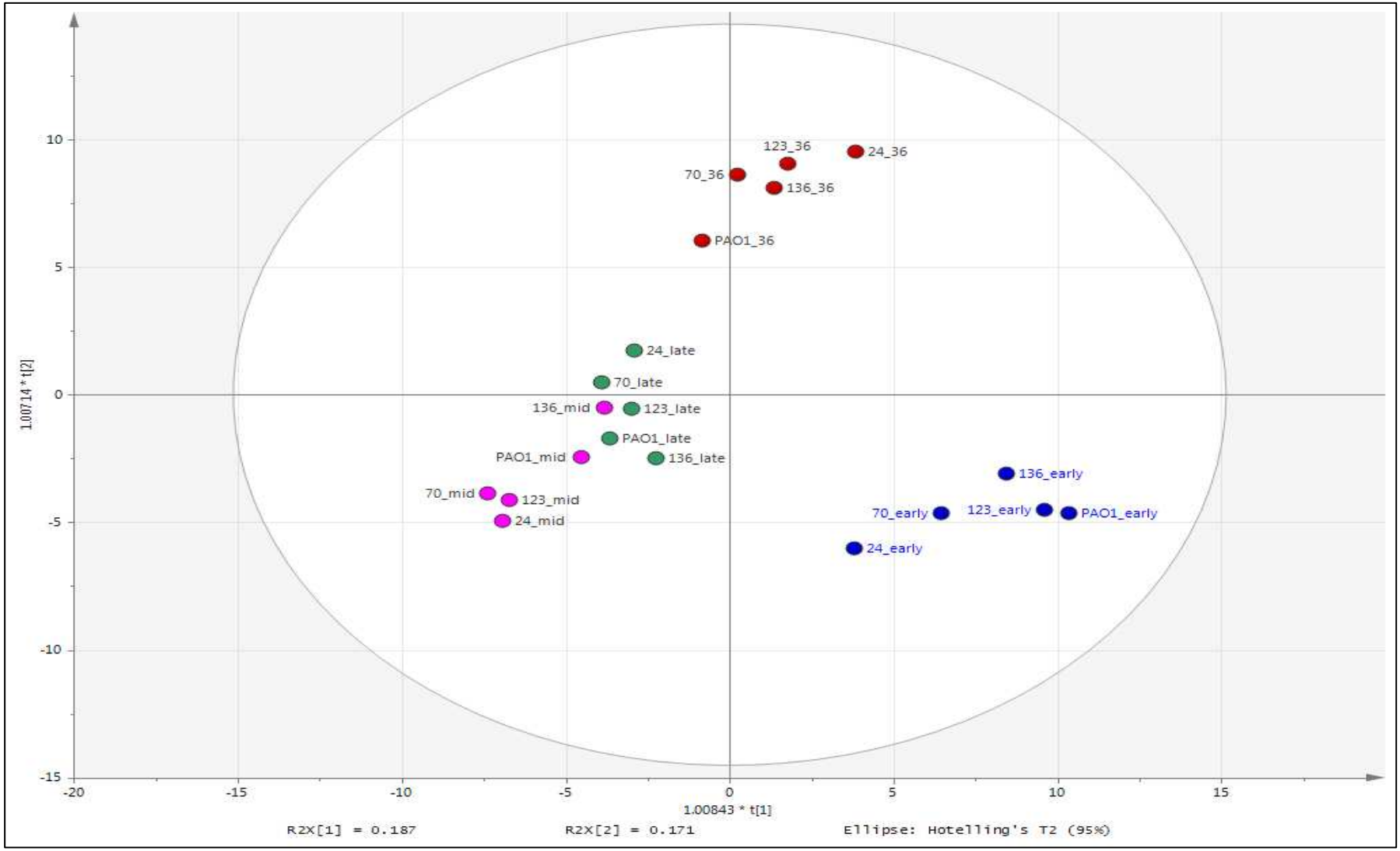
123_36
24_36
70_36
136_36
PAO1_36
24_late
70_late
136_mid
123_late
PAO1_late
PAO1_mid
136_late
136_early
70_mid
123_mid
70_early
123_early
PAO1_early
24_mid
24_early
10
5
0
-5
-10
-15
-20
-15
-10
-5
0
5
10
15
1.00714 * t[2]
1.00843 * t[1]
R2X[1] = 0.187
R2X[2] = 0.171
Ellipse: Hotelling's T2 (95%)

Figure 5.5: An oPLS-DA plot describing proposed metabolite changes for 164 significant metabolites ($p \leq 0.05$) with a CV score of ≤ 5 during pellicle formation. Lysogens were grown for ~ 3 hours, average OD_{600} 0.124 (early), ~ 5 hours, average OD_{600} 0.494 (mid) and ~ 10 hours, average OD_{600} 1.136 (late) and 36 hours to determine changes in the metabolite profile throughout the pellicle formation process.

R^2Y is 0.62 when the discriminating factor is time.

Early growth is represented by the blue data points, mid growth by the pink data points, green points represent metabolites from late growth whilst red points represent the 36 hour growth profiles.

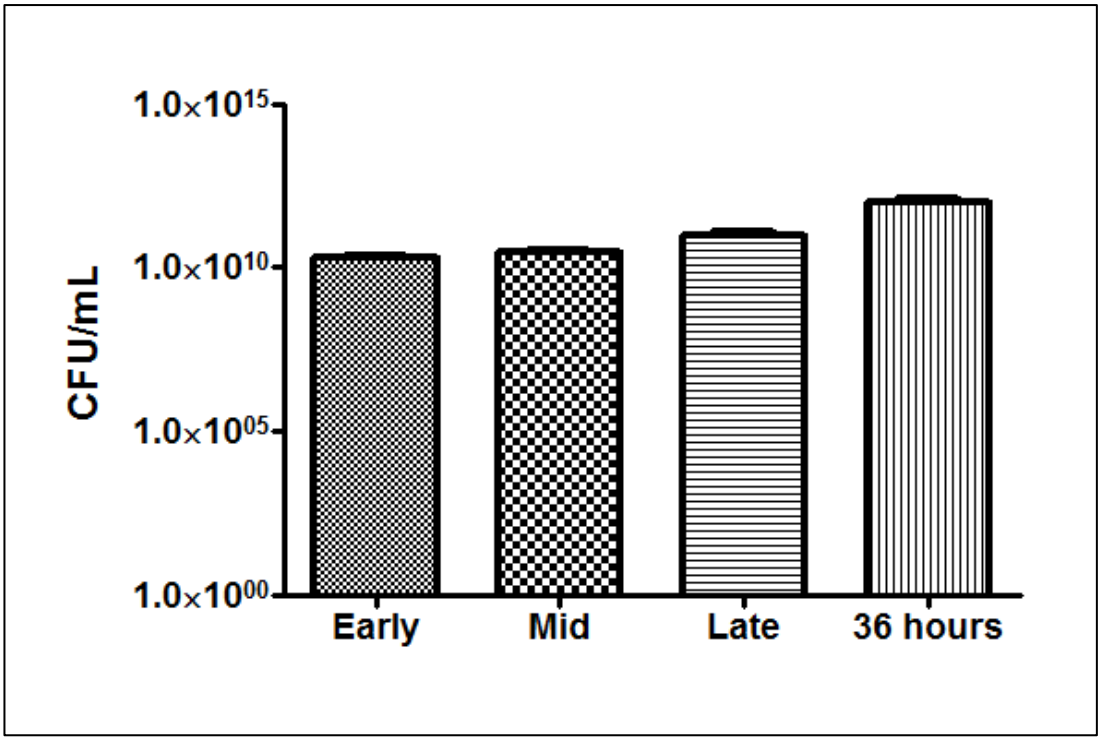

Figure 5.6: Cell count to validate the pellicle formation process. Average CFU/mL calculations for each bacterial lysogen and PAO1 at the various time points utilised for the metabolomic investigation; n = 15.

Non parametric t tests were utilised with two tailed p values shown in order to display the most discrete statistical significant data; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001.

5.3.4. Variance of importance plot (VIP) of the PLS-DA to select for proposed metabolites of interest

When characterising the metabolomic data, variance of importance plots (VIP) can be used to show which of the metabolites seen in the PLS-DA and oPLS-DA plots are significant and drive the differences observed on these models. The PLS-DA model was used for creating the VIP plot rather than the oPLS-DA. Both models have been shown to have equal predictive powers but the oPLS-DA plot has been previously shown in the literature to confer no beneficial advantage over the PLS-DA plot (Tapp and Kemsley, 2009, Ergon, 2005, Verron *et al.*, 2004, Svensson *et al.*, 2002). This however, has been argued by other researchers therefore both models were included in these results (Mahadevan *et al.*, 2008).

VIP plots show the important variables involved in determining the variation seen in the PLS-DA plot, figure 5.7. VIP plots are scored in a high to low order with confidence levels in the data being at 95 % (Tremaroli *et al.*, 2009). A VIP value greater than 1 indicates that the metabolite has been identified by PLS-DA to show a difference between the lysogenic and non-lysogenic hosts and this metabolite maybe important to function. A VIP value which is less than 0.5 indicates that the proposed metabolites are unimportant and have no potential roles in driving the variation seen on the 2 models. The area of the plot which contains metabolites with VIP values between 1 and 0.5 can be included in the analysis if the data set is small. However, due to the size of the data set in this investigation, these metabolites were not included in further analysis.

There were 41 metabolites that yielded a VIP score of greater than 1. These were subjected to further analysis and identification. Of these 41 possible metabolites, 85 % originated from positive electrospray ionisation (ESI) runs and 15 % from negative ESI runs. Upon stratification of the positive ESI runs, the

majority of the metabolites originated from < 10 BR lysogens (46 %), followed by the adult CF lysogens, the naïve PAO1, paediatric CF lysogens and > 10 lysogens (27 %, 10 %, 2 % and 0 %, respectively). When this stratification procedure was applied to the negative ESI run metabolites, it was observed that the adult CF lysogens, < 10 BR lysogens and naïve PAO1 all contributed equally. The paediatric CF and > 10 BR lysogens had no significant metabolites.

As currently there is only a limited amount of metabolism data for *P. aeruginosa*, these 41 metabolites were compared using Progenesis QI 64 bit v 4.1 and the ECMDB but only limited similarity to known metabolites was seen. There were no metabolites with an identical match when the chosen criteria were imposed upon them; *p* value ≤ 0.05, CV score ≤ 5, mass error of +/- 1. A mass error of 1 was chosen as the cut-off value as it gives more strength to the data prediction. A value closer to 1 indicates that the metabolite identified may in fact be the correct metabolite instead of being an adduct with a similar m/z ratio. In order to identify potential metabolites, it was decided to repeat the analysis process but using the human metabolome database (HMDB).

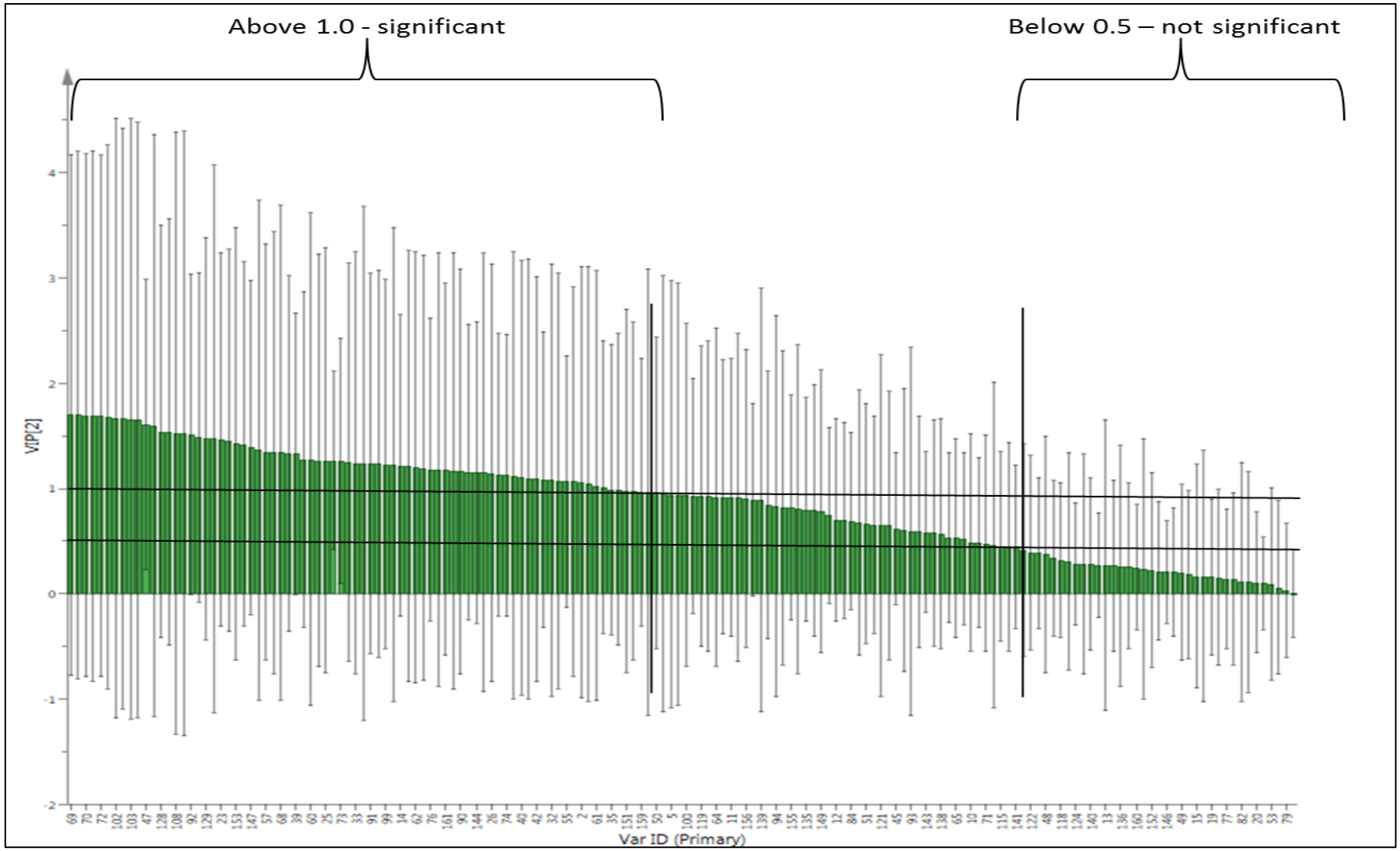
Above 1.0 - significant
Below 0.5 – not significant
V(P)[2]
Var ID (Primary)

Figure 5.7: VIP plot showing the importance of the different variables which all contribute towards the modelling of the PLS-DA plot. The x-variables or proposed metabolites that have a VIP value of above 1 are believed to be of particular interest and so further work was undertaken on these metabolites. The x-variables with a VIP value of below 0.5 are believed to have no impact on the plot and so no further work was undertaken on these samples. The x-variables with a VIP value of between 1 and 0.5 were not included for further analysis.

5.3.5. Human metabolome database (HMDB) analysis of the 41 VIP derived metabolites

As previously stated (section 5.3.4), the metabolite identification process yielded 41 metabolites which showed a significant difference between infected and uninfected cells when time and therefore, growth stage was used as the discriminator. Information on those 41 metabolites was not available when comparing to the ECMDB.

The HMDB was therefore, utilised to try and identify possible metabolites. The HMDB contains over 4.1 x 10^3 metabolite entries that are both water and lipid soluble. The database includes links to a range of metabolite identification programmes such as Encyclopaedia of Genes and Genomes (KEGG) and PubChem (Wishart *et al.*, 2013, Wishart *et al.*, 2009, Wishart *et al.*, 2007), these pathways may therefore, be orthologous to those found in *P. aeruginosa*.

The previous criteria used (*p* values ≤ 0.05, CV ≤ 5 and mass error +/- 1) were again applied to the samples with the only alteration being the metabolite identification database. Unfortunately, there were no metabolites detected using the HMDB when these stringent criteria were imposed. However, there were 5 metabolites that could be included if the mass error was raised to +/- 2. Accurate identification via MS/MS was not included in this investigation. A metabolite that is a nitroaromatic, broad spectrum antibiotic, active against both Gram negative and Gram positive bacteria appeared to warrant further investigation (Ryan *et al.*, 2011, Tangallapally *et al.*, 2007).

5.4. DISCUSSION

Over the last 10 years, there have been rapid increases in the amount of data generated via "–omic" technologies, this data is now available to characterise differences between bacteria and determine how they alter during growth (Zhang *et al.*, 2010). This study has shown subversion by phages during infection can be studied by changes in cellular metabolism and metabolite profiles. This study has also investigated the effect that time has on pellicle formation and has tried to elucidate the effect that *P. aeruginosa* phages may be exerting on their bacterial hosts at specific 'snapshot' points during pellicle formation.

To recreate the establishment of a pellicle in the presence of phage, it was observed that the addition of phage immediately after setting up the pellicle, produced pellicles that could be effectively harvested for further analysis. Infection of the culture in this manner was preferred because the phages did not need to penetrate the ECM of the establishing pellicle which increased the chance of successful phage infection. Phages have however, been proven to penetrate the bacterial cell matrix post biofilm formation through their ability to encode for hydrolases or lyases which recognise and degrade bacterial polymers in the ECM (Labrie *et al.*, 2010b, Sutherland, 1999, Sutherland *et al.*, 2004). It is also possible that phages can infect bacterial biofilms through the water channels which make up the matrix structure, which therefore, allows the phages access to the inner biofilm (Sutherland *et al.*, 2004). Whilst bacterial cells can protect themselves from phage infection through various mechanisms (section 1.6.5.), and phages have co-evolved in order to penetrate the ECM, the decision to add the mixed temperate phage lysate initially to the *P. aeruginosa* culture was to reduce any barriers which the phage may encounter whilst crossing the ECM.

The metabolism marker study shows that the addition of phage to a growing pellicle culture can increase the metabolic activity of the bacterial host, as

previously proposed by Sonkar *et al* (2012). This was determined using an XTT assay which directly linked cellular respiration and colorimetric changes, these changes were detected by spectrophotometry. Not all the alterations in the metabolism profiles were significant. This indicated that the addition of a phage doesn't provide the bacterial host cell with this evolutionary advantage under all circumstances. There seemed to be no increase in metabolism when self-phage was used in the cross infection study as the greatest differences were observed when phages originating from other *P. aeruginosa* isolates were utilised. This potentially indicates that the phages, regardless of their clinical origin are subverting their bacterial host cells and the process is not due to the phages being 'known' by the host cells.

This is one of the first studies which utilises XTT assays to determine how phages affect cellular metabolism within a pellicle community. XTT assays have previously been used to study bacterial metabolism (Cerca *et al.*, 2005) but the inclusion of bacteriophage is a novel aspect. Studying alterations in the metabolism of bacterial biofilms/pellicle has also previously been shown (Peeters *et al.*, 2008, Palomino *et al.*, 2002, Adam *et al.*, 2002), but studying metabolism alterations when a pellicle community harbours a mixed temperate phage lysate is again a novel concept.

In general for the XTT assays, it was observed that the addition of phage 24 (adult CF) led to an increase in the metabolism profiles across the bacterial hosts tested. It was also interesting to note that during the analysis of the LCMS data, the adult CF was the most disparate as a lysogen throughout growth, as shown on both the PLS-DA and oPLS-DA plots. These two findings were not unexpected as the adult CF phages in the previous chapters have been shown to be the "most evolved" and generate the most extreme profiles in terms of bacterial growth, antibiotic tolerance and infectivity profiles. This evolution is

proposed to be related to *P. aeruginosa* colonisation time but care is needed when inferring results in this manner, as there is no clinical data available for these samples to determine when the patient was initially colonised with *P. aeruginosa.*

The CF pellicles in general were more metabolically active compared to the BR pellicles and this is perceived to be a consequence of the CF phages being more infective compared to the BR phages, again this perception is based upon *P. aeruginosa* colonisation time and so the aforementioned caveat needs to be considered. This perception is enforced by work shown in the previous chapters, where the BR phages are seen to drive less extreme responses compared to the CF phages, especially when studying the growth profiles in chapter 4. Potentially, the CF phages increase the metabolism of their host cells and this allows for the maintenance of the phage within the bacterial cell. These active host cells would have replication machinery constantly driving cellular proliferation thus, allowing for the replication of the phage residing within a prophage region. The observation of an elevation in metabolism may also be due to the pellicles being thin therefore, only the active cells at the cell-surface interface may have been harvested and selected for the XTT analysis (Davies, 2003, Gjersing *et al.*, 2007). It is possible to premise that the increase in metabolism links to an increase in the amount of viable cells present in a community, so indicating that the pellicles prior to XTT addition were actively growing. The low MOI (0.1) used for the generation of the pellicles supports the constant replication of lysogenic bacterial cells and increasing bacterial numbers, which may also have led to an elevation in absorbance levels.

It was also interesting to note that the addition of the > 10 BR phage led to a decrease in the metabolism of the bacterial cultures in some pellicles. It is possible to propose that this is because the BR phages are less evolved to the

specific environment and therefore, are not providing their bacterial hosts with a selective advantage in order to increase their metabolic activity. It is possible that the addition of the phage DNA in this instance confers a burden onto the cell and so lowers its ability to respire. This may be a result of the phage integrating into an area of the bacterial chromosome which lowers respiration or blocks a key cellular process. In support of this reduction in metabolism upon the addition of a BR phage, it was seen that the lysogen which aligned closest to the PAO1 control upon LCMS analysis also contained a BR phage but in this instance it originated from the < 10 BR *P. aeruginosa* isolate. This would potentially indicate that the BR phages are not exerting large differences on their PAO1 bacterial host cells but these findings are not unexpected. This is because our previous findings have shown that the BR phages are the least evolved and the most naïve due to their new colonisation state.

oPLS-DA plots were chosen to represent the metabolomic data alongside PLS-DA plots, as these plots maximise the separation between classes based on the inputted data whilst also minimising within class variation (Zhang and Powers, 2012, Beale *et al.*, 2013). When comparing the time points on both models, it was seen that the metabolites generated during mid and late growth aligned together with minimum separation along the x-axis. This potentially demonstrates that the metabolites used to stratify the data in these models didn't change dramatically during these growth phases. The metabolites generated after 36 hours of growth align in a separate group, this group shows variation from the mid and late growth profiles due its location along the x-axis. The metabolites generated during early growth show the most variation which suggests that these metabolites are the most unique and are possibly related to a planktonic mode of bacterial growth. These LCMS findings agree with the findings of Gjersing *et al* (2007) as that study showed that metabolic profiles differ due to variations in

infection, adaptation and evolution. Gjersing *et al* (2007) showed a marked difference in NMR profiles when comparing the intracellular metabolites generated by biofilm and planktonic cells and these results could be indicative of differences in bacterial infection, evolution and adaptation. This difference is also shown in these data due to the alignment of the early (planktonic) and 36 hour (biofilm) metabolites on both the PLS-DA and oPLS-DA plots.

The data proposed by the PLS-DA and oPLS-DA plots was transposed into a VIP plot. This plot offered 41 discrete metabolites with VIP values greater than 1. These were the metabolites which drove the variation in the data and thus, the differences observed between the metabolic profiles for the naïve and lysogenic cultures. Even though these metabolites were determined to be important due to their CV scores, they were not detailed enough in order to be significant upon further progenesis analysis utilising the ECMDB. The main reason for this was that upon comparison of these data to the ECMDB, the mass error rates were not specific enough. The mass error should be +/- 1 but in some instances the mass error was 300. This made it difficult to specifically identify these metabolites and confer a putative function. The lack of a *P. aeruginosa* specific metabolome database hindered the identification process of specific *P. aeruginosa* metabolites.

In order to try and identify metabolites which were significant and also had good mass error values, it was decided to raise the mass error to +/- 2. These less stringent criteria were applied when undertaking a second round of analysis with the HMDB. When these conditions were applied, 5 metabolites were identified but the most interesting metabolite was Nitrofurazone (NTZ). NTZ has been observed to be active against some Enterobacteriaceae and Gram positive bacteria but it is in fact ineffective against *P. aeruginosa* and *Candida albicans* (Stefanides *et al*., 1976, Cunha *et al*., 2011, Brumfitt and Hamilton-Miller, 1998).

NTZ has previously been used to treat burn infections and this was of interest due to the knowledge that *P. aeruginosa* infections can cause serious complications in the treatment of burns patients (Japoni *et al.*, 2009, Church *et al.*, 2006, Altoparlak *et al.*, 2004). NTZ is also used to topically treat skin and mucosal membrane wounds, burns, infections and ulcers (Kouchak *et al.*, 2014, M Balcao *et al.*, 2013). No link towards *P. aeruginosa* naturally produced antibiotics could be drawn from the literature but NTZ intermediates and derivatives also have no activity against *P. aeruginosa*. Nitrofurantoin is an example of an NTZ derivative, this is an often under-used antibiotic which functions via interrupting growth at 3 sites within the bacterial Krebb's cycle (Cunha *et al.*, 2011, Cunha, 2006, McOsker and Fitzpatrick, 1994, Brumfitt and Hamilton-Miller, 1998). *P. aeruginosa* is naturally resistant towards this antibiotic (Brumfitt and Hamilton-Miller, 1998, Cunha *et al.*, 2011). It is interesting to consider why a phage from a clinical *P. aeruginosa* isolate derived from a < 10 BR patient (mass error + 1.57) potentially encodes for this antibiotic – could the acquisition of this antibiotic be a potential survival technique for the *P. aeruginosa* species thus, allowing *P. aeruginosa* to become predominant within the lung?

This study has emphasised the complexity of the bacterial communities and their metabolism in the lower lung. Even though no MS/MS analysis was undertaken, these data showed global differences in *P. aeruginosa* metabolite profiles when infected with phages. A confounding problem was the lack of available databases which would have allowed for more comparisons to be undertaken and could feed into KEGG pathway analysis. This would have allowed us to characterise which systems the phages subverted over time and how this linked to growth, adaptation and evolution of the bacterial host within the chronic lung. This study has shown that it is possible to undertake metabolomics on pellicle cultures of *P.*

aeruginosa and potentially identify metabolites of interest. Furthermore, this study has proposed that phages subvert their bacterial host cell via the utilisation of new processes or biological pathways.

6. METAGENOMIC ANALYSIS OF MIXED TEMPERATE PHAGE LYSATES INDUCED FROM SINGLE *PSEUDOMONAS AERUGINOSA* ISOLATES

6.1. INTRODUCTION

So far this study has focused on a culture collection of 94 *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates from cystic fibrosis (CF) and bronchiectasis (BR). Polylysogeny has been determined to in part be associated with a broader host range potential (chapter 3). The ability of phages to re-infect their originating host cell also increases as lung infections progress (chapter 3). During disease progression, the bacteria adapt certain traits which correlate to fitness increases in the chronic lung and therefore, we can hypothesise that phages may mirror this evolutionary pattern of continual interaction and evolution.

The introduction of next generation sequencing and in this instance the sequencing-by-synthesis method; offers an affordable method to look at the phage-ome of the mixed induced phage lysates isolated from each of the 94 clinical *P. aeruginosa* isolates. This chapter characterises the metagenomic profiles of the phage lysates from each clinical aetiological subgroup. It is proposed that the adult CF phages will have the most complex metagenomic profiles, which may be linked to their longevity within the chronic lung environment. A caveat relating to *P. aeruginosa* colonisation times is pertinent to mention here, the age of the patient doesn't always correlate with the length of time that a patient may have been colonised with *P. aeruginosa* for and so care needs to be taken here.

6.1.1. Culture dependent techniques

Culture dependent isolation of bacterial or viral species relies on the ability to cultivate these biological entities or having a sensitive host for the virus to infect and proliferate on within a laboratory setting. Over 70 % of bacterial species inhabiting mucosal surfaces have yet to be cultured (Barer and Harwood, 1999). The remaining 30 % whilst able to be grown in laboratory settings can be difficult to propagate (Suau *et al.*, 1999, Hayashi *et al.*, 2002, Sibley *et al.*, 2011, Han *et al.*, 2012).

CF sputum samples in hospital environments are routinely assayed for a limited but known set of bacterial species including *P. aeruginosa*, *Staphylococcus aureus* (*S. aureus*), *Haemophilus influenzae* and *Burkholderia cepacia* complex C. These culture dependent methods however, are open to detection bias (Rogers *et al.*, 2004). The selective media utilised for culturing the 'traditional' CF microbes do not support the isolation of fastidious bacteria or fungi and anaerobic species colonising the lungs (Bittar *et al.*, 2008, Belkum *et al.*, 2000). This media can also become contaminated due to the overgrowth of a 'traditional' CF bacterium such as *P. aeruginosa* (Bittar *et al.*, 2008, Belkum *et al.*, 2000). Even if bacterial species are isolated via culture dependant mechanisms, then there are often other problems involved in the identification process. For example, bacteria isolated from the chronic lung often exhibit multiple phenotypes which could potentially have an impact on the identification process (Wellinghausen *et al.*, 2005, Vaudaux *et al.*, 2006, McMenamin *et al.*, 2000, Bittar *et al.*, 2008). Culture dependent studies were not utilised in this thesis due to the potential issues that may have arisen through cultivation of *P. aeruginosa* phages and the possible lack of a receptive host. Lack of a sensitive host cell may allow some of the phage isolates to go undetected so to misrepresent the true and entire viral load of the *P. aeruginosa* isolates. Due to the large scale nature of the

investigation, time pressures of phage purification were also a key consideration that needed to be taken into account when conducting the experimental design.

6.1.2. Metagenomics

Metagenomics is based upon sequencing DNA directly from the environment without the need for prior cloning steps or PCR amplification, this technique was initially proposed by Pace and colleagues in 1985 (Pace *et al.*, 1985). The first metagenomics investigation focused on seawater, with the study aiming to link functionality to unknown microorganisms within the community (Schmidt *et al.*, 1991, Stein *et al.*, 1996, Riesenfeld *et al.*, 2004). Many environmental metagenomes have been studied including the soil (Delmont *et al.*, 2011, Rondon *et al.*, 2000), the oral cavity (Lazarevic *et al.*, 2009, Belda-Ferre *et al.*, 2012), faeces (Breitbart *et al.*, 2003, Manichanh *et al.*, 2006, Victoria *et al.*, 2009) and aquatic environments (Suttle, 2005, Suttle, 2007, Rodriguez-Brito *et al.*, 2010). Rodriquez-Brito *et al* (2010) utilised metagenomics in order to determine whether bacterial and viral taxa in aquatic environments follow the same growth patterns as seen in macro organisms. This question had been proposed previously but without the use of metagenomics and therefore, was limited by the non-cultivability of certain viruses and microbes. Their results showed that bacterial and viral populations fluctuate over time which is something that may not have been detected using culture based techniques (Rodriguez-Brito *et al.*, 2010). This population fluctuation supports the red queen hypothesis that has been previously described between bacteriophages and their bacterial hosts and supports the theory of co-evolution (section 3.3.1).

Current next generation sequencing techniques involving sequencing-by-synthesis have allowed metagenomics to become economical and have added

power through increased DNA sequencing depths. This approach generates a large amount of data relating to the genomic DNA content of an environment (Huson *et al.*, 2007). These shotgun sequencing methods allow researchers to take a 'snapshot' of a particular environment at a specific time point and due to the direct sequencing approach the entire microbiota can be investigated at once.

As this study is targeting multiple temperate phages in one *P. aeruginosa* isolate, a metagenomic approach is beneficial as it allows for the detection of all the individual phages without the need for purifying them. Now deep sequencing is possible both for phages with low titres and those that are non-infective due to lack of a known host range can be characterised. Metagenomics also allows for a function to be assigned to a metagenome. This has particular interest in this thesis which is based upon linking bacteriophages to adaptation, evolution of function within the lung and how these underlying variations in host cell functionality occur over time.

6.1.2.1. Metagenomic studies within the lungs

The human respiratory tract is a unique environment due to the constant bombardment from airborne particles including viruses, bacteria and allergens as well as antibiotic/antimicrobial treatments (Heyder, 2004). A study undertaken by Willner *et al* (2009) focused on the comparison of viruses isolated from both CF patients and non-CF individuals. In the majority of the sequences, over 90 % were unknown which is a comparable finding to other environmental viromes (Angly *et al.*, 2006, Desnues *et al.*, 2008, Dinsdale *et al.*, 2008, Willner *et al.*, 2009). Willner *et al* (2009) through metagenomics proposed that there were ~ 170 unique DNA viruses in the respiratory virome regardless of clinical aetiology.

Other viromes have been shown to have a much greater species richness for example, the ocean was found to harbour around 1.2 x 10^3 viruses and hot springs 1.4 x 10^2 viruses (Angly *et al.*, 2006, Schoenfeld *et al.*, 2008). The low species diversity in the lungs is hypothesised to be a result of both the physical and biological barriers present whilst antibiotic treatment may also further deplete the species present (Knowles and Boucher, 2002, See and Wark, 2008, Palmer *et al.*, 2005, Palmer *et al.*, 2007).

6.1.2.2. Bacteriophage metagenomics

Metagenomic studies have been undertaken on phage communities either to identify novel species or to identify specific binding regions that may influence phage longevity within a specific microenvironment (Barr *et al.*, 2013, Tariq *et al.*, 2015). Viral metagenomes have been shown to be mostly comprised of novel sequences with no known homologues (Pedulla *et al.*, 2003a, Rohwer *et al.*, 2000, Chen and Lu, 2002). Breitbart *et al* (2003) using a metagenomic approach studied the faeces from one healthy adult male. The most abundant sequences were related to bacterial species with the second most abundant being related to bacteriophages in particular; A118 of *Listeria monocytogenes*, E125 of *Burkholderia thailandensis* and bIL285 of *Lactobacillus lactis.* This study emphasised the ability to identify previously unknown bacteriophages and so it has proposed that metagenomics is an ideal tool that phage researchers should utilise in order to identify and classify novel bacteriophage genomes.

The majority of known marine viruses are phages that can influence global biogeochemical cycles (Fuhrman, 1999), genetic exchange (Paul, 1999, Breitbart *et al.*, 2002) and microbial diversity (Wommack and Colwell, 2000, Bratbak *et al.*, 1996). The viral biodiversity of these marine viruses however, is poorly

understood and the evolutionary relationship between these marine and non-marine phages is also unknown. Thus, metagenomic studies have tried to elucidate evolutionary relationships and functional similarities between these viruses (Fuller *et al.*, 1998, Hambly *et al.*, 2001, Breitbart *et al.*, 2002). Of the metagenome sequences identified in one study, the majority showed no significant similarity to previously reported sequences in GenBank (Breitbart *et al.*, 2002). This enforces the idea that metagenomics is a technique that can be utilised to identify free phage, which would otherwise remain undetected using traditional culturing based methods due to the possible inability to find a sensitive bacterial host range to propagate the virus on.

The field of DNA sequencing and its chemistry is constantly evolving in order to elucidate the entire bacterial and viral load in a population and metagenomics is becoming an increasingly common tool in unknown community studies. Novel bacterial and viral species are being identified and uploaded to GenBank on a frequent basis and this is proposed to be due to the development of low cost sequencing platforms. The development of Sanger sequencing in the 1970's led to an explosion in commercial sequencing platforms and available sequencing chemistries (Sanger, 1975, Sanger and Coulson, 1975, Gilbert and Maxam, 1973) These platforms are still being developed in order to generate platforms with the most high throughput data outputs and the lowest error rates.

In order to perform metagenomic analysis in this chapter, the Illumina MiSeq platform was utilised (Illumina, CA, USA). Illumina employs a unique indexing system which places an individual barcode for the sample within one of the sequencing adaptors. This is unique as other platforms add the barcode directly to the terminal end of the fragments (Craig *et al.*, 2008, Meyer *et al.*, 2008, Meyer and Kircher, 2010). This set up allows for a high degree of flexibility in experimental design, which is often of interest during sequencing investigations

(Meyer and Kircher, 2010). The sequencing chemistry that this machine uses is sequencing-by-synthesis and this chemistry is described in more depth in section 2.8.1.

6.2. AIMS

In chapter 3, the host range and evolution between the *P. aeruginosa* phages isolated from clinical CF and BR related isolates was linked to polylysogeny. It is also thought that genome assembly of mixed phage samples is difficult as Smith *et al* (2012) describes high levels of similarity between gene regions which would offer problems when constructing single phage genomes.

The key aim in this chapter was to compare the aetiology of infection and the clinical groupings in order to assess functionality changes in the phage metagenomes. The question of interest was 'do genes linking to survival, adaptation and evolution in the chronic lung become more prevalent as disease progresses?'. The previously mentioned caveat needs to be considered also in this instance.

Before this was attempted, a rigorous experimental design was needed to create an effective protocol for clearing contaminating bacterial chromosomal DNA from the phage viral preparations. The removal of bacterial DNA was required in order to aid downstream analysis, removing the bacterial DNA improves confidence that the regions of similarity observed are due to phages and not bacterial chromosomal DNA. In order to generate the metagenomic profiles for the phage lysates in this investigation, the samples were sequenced on the Illumina MiSeq using a 250 bp paired end library preparation. Metagenomics Rapid Annotations based on Subsystem Technology (MG-RAST) and the generation of Kyoto

Encyclopaedia of Genes and Genomes (KEGG) pathway maps were used to assign gene functions. These maps show the 'incidence' of relatedness of the DNA profiles of the phage lysates.

6.3. METHOD DEVELOPMENTS

There are a wide number of manufacturer kits and methods available to isolate total viral DNA, although for analysis of the virome via metagenomics DNA needs to be free from bacterial contamination. Prior to the various DNA purification methods being used, the phages were induced from the bacterial chromosome using fluroquinolone antibiotic, Norfloxacin (section 2.3.3.).

6.4. RESULTS

6.4.1. PEG8000 purification of 94 mixed temperate phage lysates originating from clinical *P. aeruginosa* isolates

PEG8000 was used to precipitate the virus DNA using the method proposed by Sambrook *et al* (1989). Preliminary work involved the use of *E. coli* ϕ 24_B to determine how effective the process was for clearing contaminating bacterial chromosomal DNA away from the lysate. PCR amplification of the *Q* gene (late gene antiterminator), was undertaken on the phage samples post PEG8000 purification in order to determine whether a phage was present or absent (435 bp) (Smith *et al*., 2007). PCR amplification did show the presence of the phage *Q* gene after PEG8000 purification, showing that this process was indeed successful for purifying phages.

When the same method was applied to the *P. aeruginosa* phage lysates however, it was decided to utilise PCR amplification of the V3 region of the 16S rRNA gene to ascertain the level of bacterial chromosomal contaminaton (Muyzer *et al.*, 1993, Chakravorty *et al.*, 2007). The V3 region post PCR amplification generates a product on an agarose gel of 195 bp (Chakravorty *et al.*, 2007), figure 6.1. A low intensity band was detected on a 1 % agarose gel after PCR amplification and this lead to the QUBIT 2.0 (Life technologies Ltd), being utilised in order to determine the exact amount of DNA present in the samples. The QUBIT 2.0 showed that the DNA concentration was too low for further downstream applications.

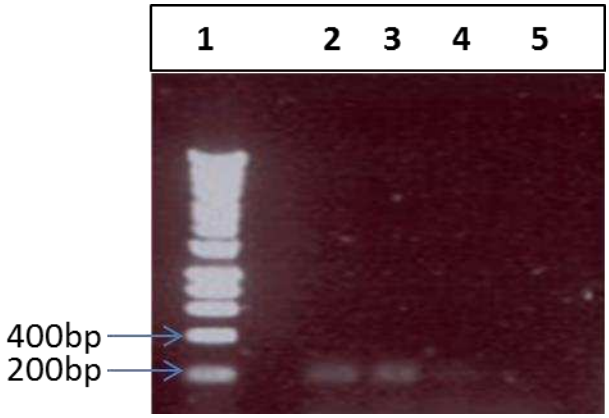

Figure 6.1: A 1 % agarose gel showing amplification of the 16S rRNA gene following PEG8000 purification Lane 1 is Hyperladder 1, lanes 2 – 5 are various phage lysates subjected to PCR amplification post PEG8000 analysis. The low intensity band is apparent in this gel image.

6.4.2. QIAGEN QIAmp MinElute Viral Spin Kits use in the purification of 94 mixed temperate phage lysates originating from clinical *P. aeruginosa* isolates

The QIAGEN QIAmp MinElute Viral Spin Kit was next selected to isolate the phage DNA isolation in this investigation, as the manufacturer has previously shown the effectiveness of this kit in purifying viral DNA.

In order to clear the contaminating bacterial chromosomal DNA from the phage lysate, varying amounts of TURBRO DNAse and RNAse cocktail were used (Life technologies) and different incubation periods prior to running the phage lysate through the QIAGEN kit. It was proposed that varying the levels of DNAse and RNAse would clear the contaminating bacterial DNA from the lysate whilst leaving the phage DNA intact due to protection by the phage capsid.

DNase has been used in the molecular biology field to clear genomic DNA contamination and it works by digesting the DNA from dead bacterial cells. The bacterial cells in this investigation are presumed to be dead as they have just been subjected to processing by the QIAGEN QIAmp MinElute Viral Spin Kit (Villarreal *et al.*, 2013, Wang *et al.*, 2002). However, DNAse is not effective at clearing genomic DNA from intact bacterial cells and so this is why downstream removal of the DNA was required (Villarreal *et al.*, 2013). The increasing volumes of DNAse and RNAse were added into 1 mL of induced phage lysate in increasing amounts and incubation at 37 ℃ was performed with the time being altered from between 30 minutes to 4 hours. A 1 % agarose gel image showing the presence of a 16S rRNA gene post DNAse/RNAse and the QIAGEN kit treatment is shown in figure 6.2.

2 U/L of DNase and and 500 U/L of RNase A and 20,000 U/L of RNase T1 was selected for future work and an incubation time period of 30 minutes at 37 ℃.

This time point was chosen as there were no discernible differences between the results generated when using larger volumes of DNAse and RNAse with longer incubation times. Due to the high level of contaminating bacterial chromosomal DNA, detected by PCR after the use of the QIAGEN kit even after the varying amounts of DNase and RNase, it was decided that another method was needed in order to generate the purest phage DNA.

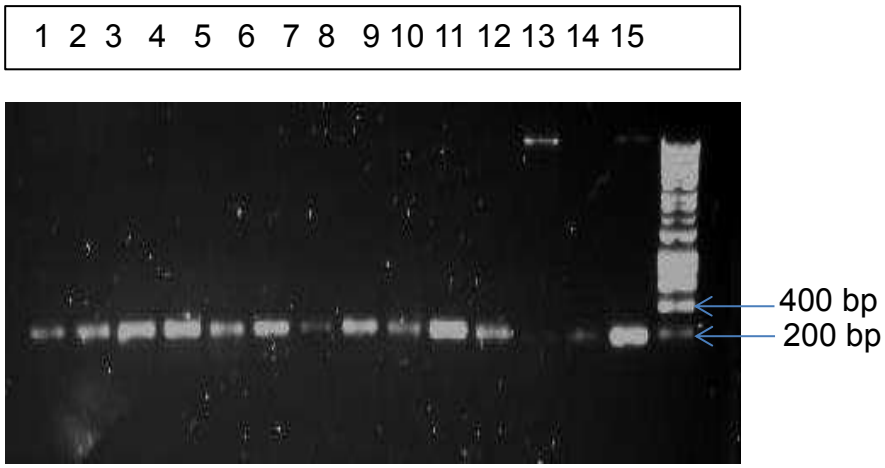

Figure 6.2: 1 % agarose gel image showing 16S rRNA gene PCR following varying treatments with DNAse and RNAse. Lane 1 treatment with: 10 µL of DNAse and RNAse for 1 hour, lane 2 treatment with: 9 µL of DNAse and RNAse for 1 hour, lane 3 treatment with: 7 µL of DNAse and RNAse for 1 hour, lane 4 treated with: 5 µL of DNAse and RNAse for 1 hour, lane 5 treated with 3 µL of DNAse and RNAse for 1 hour, lane 6 treated with: 1 µL of DNAse and RNAse for 1 hour. Lane 7 treated with:10 µL of DNAse and RNAse for 30 minutes, lane 8 treated with: 9 µL of DNAse and RNAse for 30 minutes, lane 9 treated with: 7 µL of DNAse and RNAse for 30 minutes, lane 10 treated with: 5 µL of DNAse and RNAse for 30 minutes, lane 11 treated with: 3 µL of DNAse and RNAse for 30 minutes, lane 12 treated with: 1 µL of DNAse and RNAse for 30 minutes. Lane 13 is the negative control, lane 14 is the positive control (PAO1) and lane 15 is Hyperladder 1.

6.4.3. NORGEN Phage DNA Isolation Kit use for the purification of 94 mixed temperate phage lysates originating from clinical *P. aeruginosa* isolates

Due to the problems encountered previously with both the PEG8000 purification and the QIAGEN kit, the NORGEN Phage DNA Isolation Kit (Geneflow Limited, Lichfield, UK), was selected in an attempt to clear the contaminating *P. aeruginosa* chromosomal DNA from the phage lysate. PCR amplification of the V3 region of the 16S rRNA gene was again undertaken in order to determine the effectiveness of this DNA isolation kit (Muyzer *et al.*, 1993). A PCR product was still present at 195 bp (Chakravorty *et al.*, 2007). Upon visualisation of the 1 % agarose gel, a band which showed a lower intensity in brightness was detected so indicating through a semi-quantitative observation, that this kit was the most efficient at clearing the *P. aeruginosa* chromosomal DNA compared to the two previously described methods.

Two elutions were undertaken using the NORGEN kit in order to increase the DNA yield present. The QUBIT 2.0 was again utilised in order to determine the DNA level present within a sample; one sample post NORGEN processing and two elutions possessed a DNA level of 0.495 ng/µL. This was suitable for downstream DNA analysis. It was decided to use this kit for the purification of the 94 clinical *P. aeruginosa* phage lysates and to discount any remaining bacterial DNA contamination via bioinformatics. The QUBIT 2.0 values for the mixed temperate phage lysates are detailed in appendices 8 and 9. PCR amplification of the 16S rRNA gene is shown in figure 6.3 and it shows the lack of detectable bands in the NORGEN treated phage lysates thus, indicating that the contaminating bacterial chromosomal DNA had been cleared from the sample.

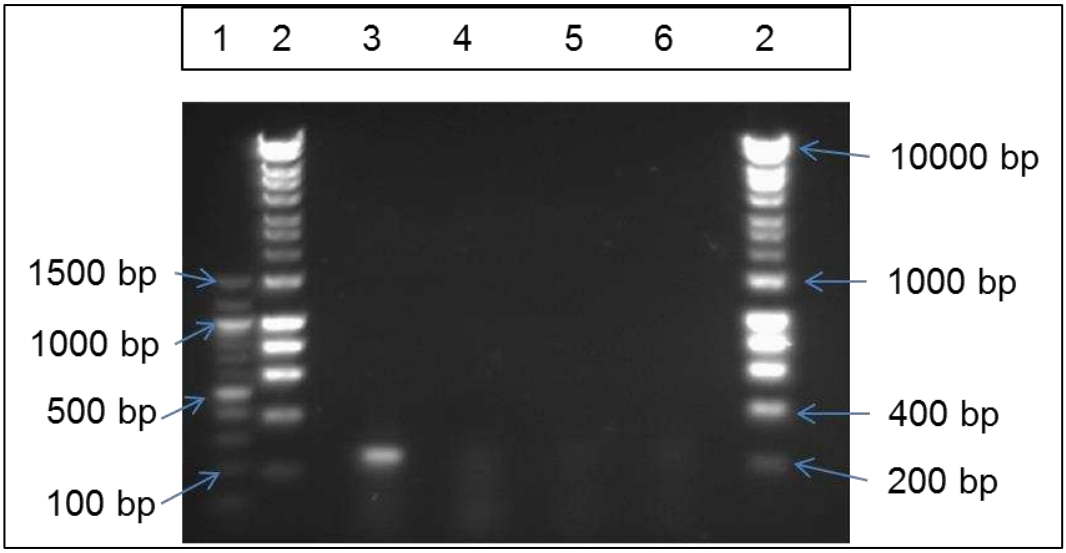

Figure 6.3: 1 % agarose gel showing amplification for the 16S rRNA gene (195 bp) after using the NORGEN phage DNA isolation kit. Lane 1 – Quick load ladder, lane 2 – Hyperladder 1, lane 3 – positive PAO1 control, lane 4 – negative control, lanes 5 and 6– paediatric CF phage DNA post use of the NORGEN phage DNA isolation kit.

6.4.4. Metagenomics Rapid Annotations based on Subsystem Technology (MG-RAST) analysis of 92 mixed temperate phage lysates originating from clinical *P. aeruginosa* isolates

Metagenomic analysis was undertaken on the 92 mixed temperate phage lysates generated from the clinical *P. aeruginosa* isolates post sequencing on the Illumina MiSeq platform; the number of reads generated for each sample are detailed in appendix 10. 2 samples were removed from downstream applications due to poor sequencing data (1 < 10 BR and 1 > 10 BR). In order to remove any remaining bacterial chromosomal DNA that persisted following NORGEN kit application, the Khmer bioinformatics toolkit was used (Brown *et al.*, 2012). As the bacterial chromosomal DNA is sheared in part through DNAse, any remaining chromosome looked like an error Khmer which generated an individual error peak that could be removed via bioinformatics analysis from the sample (method in preparation for journal submission).

The reads generated by the Illumina MiSeq were uploaded onto the Metagenomics Rapid Annotations based on Subsystem Technology (MG-RAST) website to allow for metagenomic analysis. 82 of 92 total samples were processed on MG-RAST, 10 samples fell below the required cut off values stipulated by MG-RAST (5 > 10 BR and 5 < 10 BR).

Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways were generated and the samples were split into aetiological subgroups according to their associated clinical data (paediatric CF, adult CF, < 10 BR and > 10 BR). This was done in order to allow for more pertinent observations to be drawn. The KEGG pathways are shown in figure 6.4. These pathways allowed identification of the 'incidence' of DNA sequences which shared similarity to a known metabolic pathway, for each of the clinical aetiological groups. Figure 6.4 describes the KEGG atlas maps for the stratified phage metagenomes. Similar

regions are illuminated in all the maps so potentially indicating that these relate to essential cellular functions. It was also apparent when studying these maps that the < 10 BR map showed the lowest number of gene targets for putative functionality, this could be related to the naivety of these phages however, the previously mentioned caveat needs considering alongside this data. This naivety has been shown in the previous chapters so this result is not unexpected. All the associated raw KEGG derived values are shown in appendix 11. Tariq and Everest *et al* (2015) have previously published the figures for both the KEGG pathways and the images conferring a putative function for each of the clinical metagenomes.

Figure 6.5 details the increase in putative functionality; these related hits were seen to increase with patient age/disease severity. The highest numbers of putative identifications were seen in the phage metagenomes from the adult CF and > 10 BR patients. The < 10 BR phage metagenomes display the lowest number of putative functionality hits which may correlate to the naivety of this particular phage and *P. aeruginosa* cohort.

Figure 6.6 stratifies the metagenomic dataset according to defining areas of physiology on the KEGG atlas whilst also stratifying the data according to the specific clinical aetiologies. KEGG pathway analysis allows for identification of specific gene regions and these identifications can be linked to functional pathways related to both metabolism and signalling. Metagenomic analysis has shown that functionality increases in complexity in accordance with disease progression. This supports previous findings in this thesis which have shown that the adult CF phages are the most infective and that this infectivity may be due to an additive effect of multiple phages residing within a *P. aeruginosa* genome. However, as there is no clinical data regarding the initial colonisation time of each patient with *P. aeruginosa* then care needs to be taken when drawing

conclusions such as these. Previous work has also shown that the > 10 BR phages are the most infective in the BR cohort. Again, this is perceived to be a result of elevated complexity due to disease progression and longevity. The previously mentioned caveat should also be applied in this instance prior to conclusions being drawn.

Due to the nature of the KEGG atlas maps being utilised for this analysis, it is not possible to determine the amount of hits relating to a particular pathway though. The analysis just indicates that the phage metagenomes under investigation share some similarity with specific pathways and genes located on the KEGG atlas. The results are just an indication of the possible pathways present/absent in the various phage metagenomes. The metagenomes are stratified according to the clinical aetiological data in order for trends and patterns to be observed which may be indicative of the phages residing in the *P. aeruginosa* populations isolated from the different patient subgroups.

Glycan biosynthesis and metabolism is elevated in the most evolved phage metagenomes, showing that these phages may have had to evolve in order to offer the bacteria the ability to metabolise glycans to allow for persistence within the chronic lung. This finding has been previously shown by Simpson *et al* (2015) this study showed that phage harboured a large reserve of glycan binding proteins and hydrolysing proteins which may provide the host with an evolutionary advantage and aid persistence within the lung (Simpson *et al.*, 2015).

Another putative KEGG functionality hit that follows this pattern is mechanism of cofactors and vitamins. Again, this suggests that complex phages may have evolved in order to metabolise additional metabolites, which may aid the long term colonisation and persistence of phages within the chronic lung.

The BR phage metagenomes showed higher incidence for sequences relating to energy metabolism compared to the CF metagenomes. Upon analysis of the KEGG pathway map, (figure 6.4) the pathways that are involved in energy metabolism were seen to be different between the BR and CF phage metagenomes. So this would indicate that the phages from the two clinical aetiologies are utilising different energy metabolism pathways. This finding is in contrast to the reduced growth profiles seen for the BR lysogens in chapter 4, although this may relate to the environment that the bacteria and phage are co-evolving in.

Another putative functionality pathway that is enriched in BR phage metagenomes is the biosynthesis of other secondary metabolites. It is possible that these metabolites are involved in the changes in antibiotic tolerance seen in chapter 4. Therefore, these metabolites may potentially aid the phage's conversion and long term survival of their bacterial host. As seen in chapter 5, a broad spectrum antibiotic, NTZ was isolated from a BR patient and this may be an example of secondary metabolite which aids *P. aeruginosa* survival and colonisation within the BR lung.

Sequences identified which are related to xenobiotic degradation were seen at the highest levels in the adult CF phage metagenomes, possibly showing that the phages have evolved functions to degrade xenobiotic's as a consequence of the constant bombardment of the CF lower lung with antimicrobials and antibiotics. Xenobiotic degradation is the breakdown of xenobiotics which include antibiotics and antimicrobials. It may be advantageous for the phages to harbour functions relating to this degradation as it will aid their persistence within the CF lung whilst also aiding *P. aeruginosa* persistence within the CF lung.

Figure 6.6 also shows equal levels of sequences relating to lipid metabolism in all the phage metagenomes with the exception of the < 10 BR phage. This may be explained by the naïve nature of the < 10 BR phages. This pattern is also seen in carbohydrate metabolism. Interestingly, the proposed less adapted < 10 BR phage metagenomes identified no sequences homologous to amino acid metabolism.

Metabolism of Terpenoids and Polyketides is low in all the phage metagenomes, so potentially showing that these secondary metabolites may not be utilised by phages and bacteria when surviving and evolving in the chronic lung.

Sequences relating to nucleotide metabolism were lowest in the > 10 BR phage metagenomes. This is considered unusual, as we would expect an elevated complexity in this later stage disease. These phages may however, utilise different pathways or these phages are totally dependent upon host cell machinery for their replication.

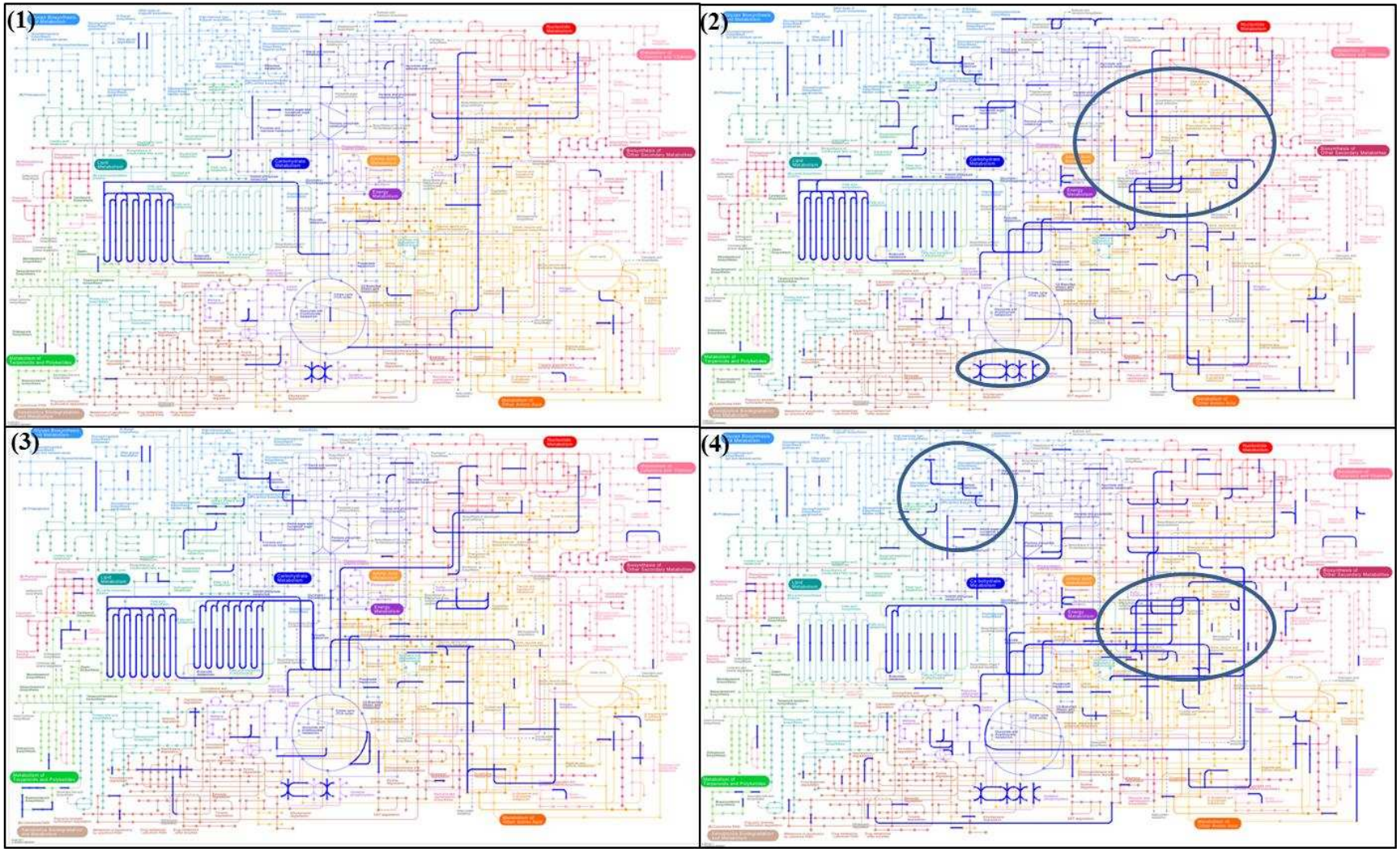
(1)
(2)
(3)
(4)

Figure 6.4: KEGG pathway maps for the 82 phage metagenomes stratified according to clinical aetiologies. The pathways are generated via the KEGG function on MG-RAST. All the pathways indicated show regions of DNA similarity between the phage metagenomes and known functional pathways on the KEGG atlas.

The blue lines indicate when a region of similarity has been observed between the phage metagenomes and the KEGG pathway. The circled area's indicate where large differences are observed between the phage metagenomes when studying paediatric CF v's adult CF and < 10 BR v's > 10 BR.

Panel 1 describes the metagenomes for the paediatric CF phages, panel 2 describes the metagenomes for the adult CF phages, panel 3 describes the metagenomes for the <10 BR phages, and panel 4 describes the metagenomes for the >10 BR phages.

The adult CF phage metagenomes have an increase in pathway similarities for amino acid metabolism and energy metabolism particularly oxidative phosphorylation, these regions are not apparent in the paediatric phage metagenomes so indicating a possible evolutionary event has occurred. The > 10 BR phage metagenome is more enriched in pathways with similarities to amino acid metabolism and carbohydrate metabolism as described by the highlighted areas on the KEGG pathway map.

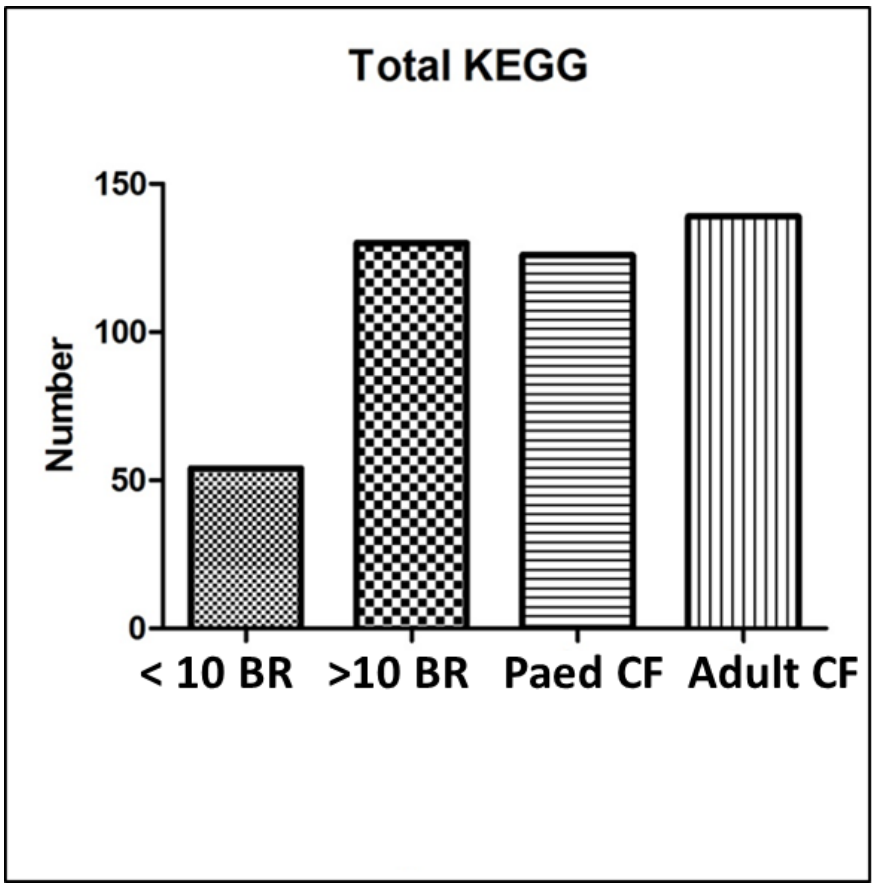

Figure 6.5: Total KEGG putative function identifications derived for the phage metagenomes when stratified according to clinical aetiology. Each putative functional pathway is represented as a single identification regardless of the amount of times that the putative function may have been conferred in each of the clinical subgroups (Tariq *et al.*, 2015). Number in this figure refers to the number of identifications observed for each clinical subgroup, exact numbers are not possible to elucidate however.

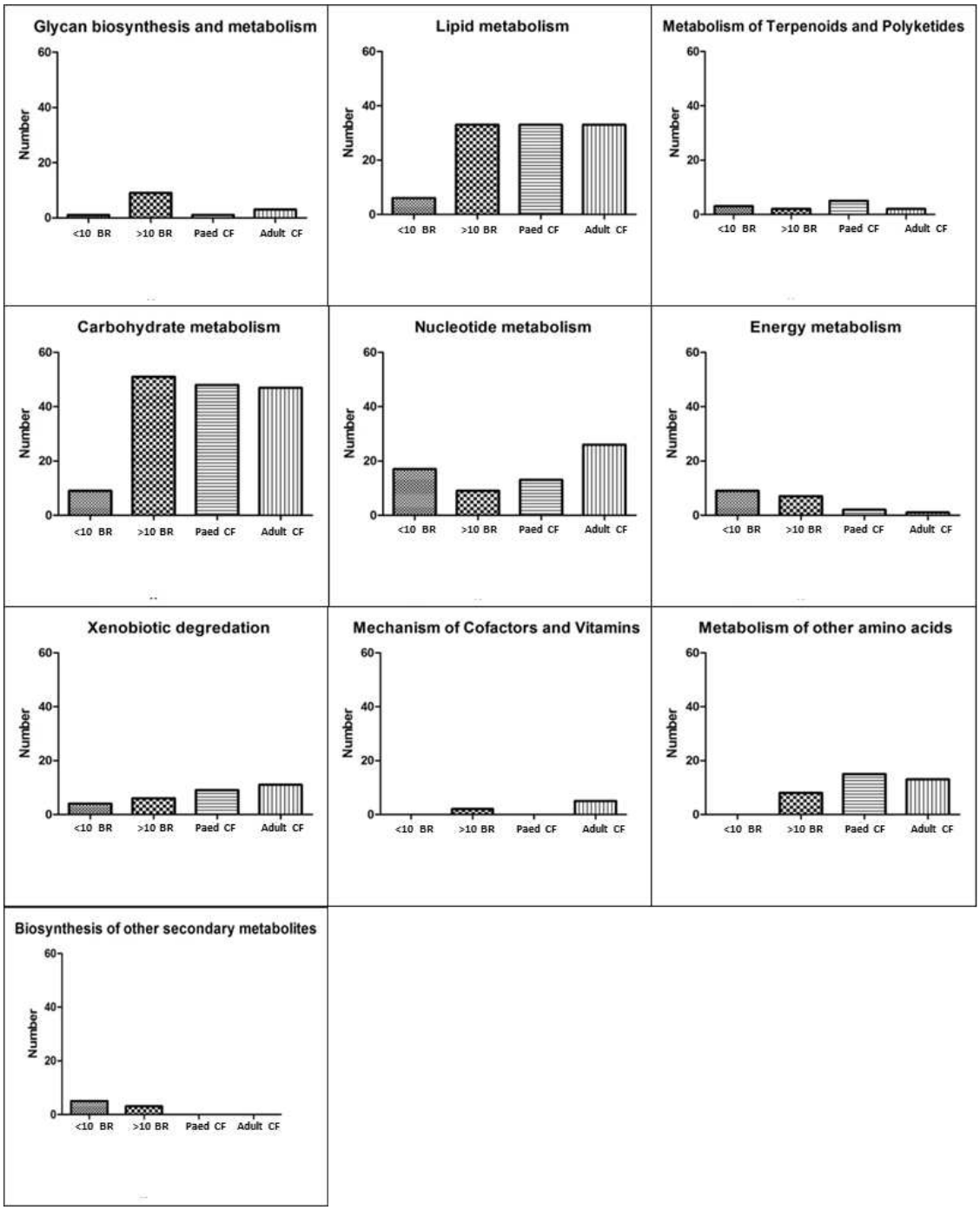
Glycan biosynthesis and metabolism
Lipid metabolism
Metabolism of Terpenoids and Polyketides
Carbohydrate metabolism
Nucleotide metabolism
Energy metabolism
Xenobiotic degredation
Mechanism of Cofactors and Vitamins
Metabolism of other amino acids
Biosynthesis of other secondary metabolites
Number
0
20
40
60
<10 BR
>10 BR
Paed CF
Adult CF

Figure 6.6: Incidence of amino acid similarity to KEGG functional pathways generated through MG-RAST. The functional pathways are characterised according to their location on the KEGG atlas and the data is also further subdivided according to the clinical aetiologies of the phage metagenomes. Each putative functional pathway is represented as a single identification regardless of the amount of times that the putative function may have been conferred in each of the clinical subgroups. The functional information inferred from these KEGG pathways can be used to confer possible functionality differences between the phage metagenomes and relate these back to information relating to the various clinical aetiologies (Tariq *et al.*, 2015). Number in this figure refers to the number of identifications observed for each clinical subgroup for each section of the atlas map, definitive numbers of identifications can't be concluded from this data though.

6.5. DISCUSSION

Various DNA purification techniques were undertaken in order to generate the purest and highest quantity DNA yield possible for the phage lysates involved in this investigation. The NORGEN phage DNA isolation kit was eventually chosen after assessing two other DNA extraction protocols for their ability to remove contaminating *P. aeruginosa* chromosomal DNA. This contamination was determined via PCR amplification of the V3 region of the 16S rRNA gene of *P. aeruginosa* (Muyzer *et al.*, 1993, Chakravorty *et al.*, 2007).

The original PEG8000 method of purification has been previously used to purify phage DNA. Rooks *et al* (2010) showed that the *Q* gene could be amplified after serial dilutions of purified ϕ 24_B phage lysates post PEG8000 purification. The amplification of this gene following multiple serial dilutions showed that the PCR process was as sensitive as a traditional plaque assay for identifying individual 24_B phage particles. This finding by Rooks *et al* (2010) supports the QUBIT 2.0 read out value of 0.000 ng/µL as it shows that the phage can be amplified even when the amount of the phage DNA present is negligible. These two observations would lead to two hypotheses being drawn. Firstly, that PEG8000 can purify phage DNA but at incredibly low levels and secondly, that the PCR amplification of the *Q* gene was dependant on the sensitivity of the PCR assay rather than large amounts of phage DNA being present post PEG8000 processing (Rooks *et al.*, 2010).

The PEG8000 method initially was developed for use with high titre phage stocks whereas, this investigation was based upon phage induction rates which may produce suboptimal phage titres (Sambrook *et al.*, 1989). Plaque purification and amplification could not be utilised in this investigation as the mixed phage community was under investigation and plaque purification focuses upon single phage purifications. These assays also require a sensitive bacterial host to

propagate on. Any remaining bacterial chromosomal DNA was negated from the culture post sequencing via the use of the Khmer toolkit (Brown *et al.*, 2012, Tariq *et al.*, 2015). This toolkit removes error khmers which appear as low level sheared DNA and thus, can be removed from the sample.

After DNA isolation and sequencing on the Illumina MiSeq (NU-Omics, Northumbria University at Newcastle, UK), the DNA was subjected to metagenomic analysis. No single gene is common to all viral species as previously mentioned - so metagenomics was utilised in order to potentially identify and confer functionality to all the phage particles present in the lung microbiome (Edwards and Rohwer, 2005). Tariq and Everest *et al* (2015) reported the first use of metagenomics to identify inducible mixed temperate phage communities induced from *P. aeruginosa* isolates which colonised the lungs of CF and BR patients; some of the data is presented here. MG-RAST was used to undertake the analysis on the metagenomes of these mixed temperate phage lysates. Upon the generation of KEGG atlas maps, there was a clear observation that the complexity of the metagenomes related to an increase in patient age (CF) or length of time since diagnosis (BR).

The finding that the adult CF phage metagenomes had the highest number of putative KEGG pathway incidences supports previously described work in this thesis, which has shown that the CF phages are the most infective and capable of causing infection in a broad *P. aeruginosa* host range (chapter 3). It is possible to premise that this previously described elevation in host range and infectivity may be linked to the phage metagenome. KEGG pathways can also be used to confer possible functionality to DNA sequences which exhibit similarity to known metabolites and metabolic pathways (Tariq *et al.*, 2015).

KEGG pathways in this instance were used to identify specific functional traits that may link the metagenomic profiles of the phages to disease severity and progression. As disease progresses in both CF and BR patients, an increase in DNA incidences relating to the KEGG atlas section; glycan biosynthesis and metabolism, was seen in the mixed phage metagenomes. Due to mucociliary defects and *CFTR* mutations (Knowles and Boucher, 2002, Matsui *et al.*, 1998, Pilewski and Frizzell, 1999, Holmén *et al.*, 2004), the CF lung contains a large amount of human produced mucins which are covered in glycans. Mucins are massive glycoconjugates that in CF patients are composed of ~ 80 % carbohydrate and these mucins form O-linked glycans. Mucus and the associated mucins in healthy people are much less complicated and contain fewer cellular components (Moniaux *et al.*, 2001, Holmén *et al.*, 2004, Rubin *et al.*, 1990, Voynow *et al.*, 2006).

P. aeruginosa binds to mucin glycans via lectins specifically PA-IL and PA-IIL which bind to galactose and fucose/mannose glycoproteins, respectively. PA-IL is a 51 kDa protein (Gilboa-Garber *et al.*, 1972, Winzer *et al.*, 2000) and PA-IIL is a 47 kDa protein (Gilboa-Garber, 1986, Gilboa-Garber *et al.*, 2000, Winzer *et al.*, 2000). These lectins regulated by the quorum sensing system in *P. aeruginosa* and they are co-expressed with other virulence factors. Lectins can therefore, be proposed to be virulence factors due to their ability to bind to carbohydrates and so retain *P. aeruginosa* within an environment (Gilboa-Garber, 1983, Jørgensen *et al.*, 1999, Gilboa-Garber, 1997, Winzer *et al.*, 2000).

It is possible to propose that phage metagenomes have evolved functions relating to glycan biosynthesis in order to elevate the potential binding sites available for *P. aeruginosa* lectins therefore, promoting bacterial longevity in the lower lung along with the associated phage longevity and survival. Winzer *et al* (2000) stated that cell density and the age of the bacterial culture altered glycan

synthesis, so it is pertinent to mention that the phage metagenomes which were the most enriched for putative functionality hits relating to glycan biosynthesis were observed in the phages originating from adult CF and > 10 BR patients. The observation of putative functions in the phage metagenomes relating to glycan biosynthesis may show that phages are involved in driving the generation of glycans in the lower lung thus, providing the bacterial-phage communities with a selective advantage in order for them to persist within the lung microbiota (Tariq *et al.*, 2015).

In contrast to the BR phage metagenomes, the CF phage metagenomes were completely devoid of incidences relating to 'metabolism of other secondary metabolites'. This may indicate that the phages colonising the CF lung are incredibly well adapted to survive within this environment and do not require other metabolites aside from those which are found within this community.

Through utilising KEGG pathways and by conferring putative functions to the metagenomes, it was observed that there was an elevation in the xenobiotic degradation atlas class which again related to an increase in disease severity in CF and BR patients. Winstanley *et al* (2009) showed that *P. aeruginosa* isolates originating from the CF lung had a fluctuating antibiotic resistance profile and this is supported by these data presented in this thesis. Variation in *P. aeruginosa* antimicrobial resistance has been shown in numerous studies and they have also shown how sub-optimal antibiotic treatment can also change the antimicrobial resistance profiles of *P. aeruginosa* (Mowat *et al.*, 2011, Ashish *et al.*, 2013, Wright *et al.*, 2013). These data showed that the adult CF phage metagenomes exhibited the highest putative functionality hits relating to xenobiotic degradation. It is possible to premise that due to the constant bombardment of antibiotics, phage metagenomes have evolved to encode for resistance to antibiotics thus, enhancing their survival potential in this adverse niche. This finding also relates

to data presented in chapter 4 where it was seen that the CF lysogens exhibited greater antibiotic tolerance results in respect to the naïve PAO1 host thus, proposing that the phage metagenome alone may be influencing this alteration in tolerance.

This elevation in complexity in the phage metagenomes could elucidate that extensive interplay is occurring between phage and bacterial communities within the chronic lung. The increase in complexity relating to disease progression could potentially indicate that adaptation is occurring in 'real time' within the lung. The phage metagenomes may have therefore, evolved in order to only harbour beneficial selective advantages and have lost the more costly genomic functions. The work described by Tariq and Everest *et al* (2015) and presented here shows that adaptation is occurring in phage metagenomes alongside their *P. aeruginosa* hosts.

7. DISCUSSION

Microbial disease is multi-factorial and therefore, multiple approaches need to be utilised to try to understand the biology of disease and how it progresses. Through environmental changes in the lung from both a congenital and acquired chronic lung disease, there are progressive rounds of inflammation and the continuous generation of scar tissue which inhibits normal mucocilliary action. These changes and a constantly evolving mucus-rich environment means that bacteria need to adapt to in order to stay in this niche. Opportunistic bacteria like *Pseudomonas aeruginosa* (*P. aeruginosa*), readily colonise the Bronchiectasis (BR)/ Cystic Fibrosis (CF) lung as mentioned in section 1.4.

The continuing interaction between bacteriophages and their bacterial hosts provides variation within this environmental niche either due to temperate phage infection and their integration into the bacterial chromosome or through phage's ability to drive recombination events in the lung microbiota via single stranded DNA recombinases which are readily carried by lambdoid-like phages (Brockhurst *et al.*, 2005). This interaction is thought to be antagonistic but it is in some way also symbiotic, as each biological entity evolves and adapts within the chronic lung, this is an example of the red queen hypothesis occurring within the lung microbiota (Stern and Sorek, 2011, Van Valen, 1973, Lenski and Levin, 1985, Schaffer and Rosenzweig, 1978, Dawkins and Krebs, 1979). The bacterial hosts evolve in order to overcome the threat of phage infection through the generation of alternative phage adsorption sites (section 1.6.1.1.) or through adaptation to overcome host defence systems (section 1.6.5.). The phages can also develop new strategies to increase the likelihood of subsequent phage infections through the subversion of bacterial host defence systems. This increase in environmental fitness of the bacterium offers negative clinical

outcomes for the patient as the *P. aeruginosa* infections become increasingly difficult to treat (Hart and Winstanley, 2002).

The aim of this research was to characterise the role that temperate bacteriophages play in the progression of CF and BR. The temperate bacteriophages involved in this investigation were induced from 94 clinical *P. aeruginosa* isolates [paediatric CF patients (n = 10), adult CF patients (n = 37), < 10 BR patients (n = 17) and > 10 BR patients (n = 30).] This thesis focused on 4 separate areas of biology in order to look at the interactions of phages and their *P. aeruginosa* hosts, for ease the data has been split into 4 subgroups according to patient data.

Chapter 3, focused upon the interaction between mixed *P. aeruginosa* phage lysates induced from single bacterial isolates and how they interacted with the complete panel of *P. aeruginosa* clinical isolates. This was completed through the use of a large scale cross-infection study, which is the largest of its kind mentioned in the literature for *P. aeruginosa*. A key driver of this chapter was to determine how the infectivity profiles of the phages and the sensitivity of *P. aeruginosa* isolates towards phage infection altered alongside disease progression. However, disease progression doesn't always link with an increase in *P. aeruginosa* colonisation time.

This work is novel for two reasons, firstly due to the large scale nature of the investigation and secondly, because it utilised mixed temperate phage communities. These mixed phage communities are a cell's entire repository of viruses that can be induced and infect other bacterial isolates. Using the mixed temperate bacteriophage load may produce results which are more indicative and representative of the chronic lung and so therefore, more weight can be given to the findings presented in chapter 3.

It was determined that the phages isolated from the older patients (adult CF and > 10 BR) had greater infectivity profiles compared to the more naïve *P. aeruginosa* isolates. When investigating bacterial sensitivity to phage infection, it was observed that similar sensitivity profiles were seen for the *P. aeruginosa* isolates regardless of the clinical origin of the phage lysate. This may indicate that a complex co-evolution is occurring which could indicate that the bacterium and phage are both equally infective and susceptible thus, ensuring the survival of both entities within the chronic lung environment. Buckling and Rainey (2002) showed antagonistic co-evolution occurring between bacteria and phage, this study is supported by these data presented in chapter 3.

Chapter 3 also presented some of the data published in Tariq and Everest *et al* (2015). It was shown that the phages isolated from *P. aeruginosa* isolates originating from the later disease stages had the greatest capabilities to re-infect their originating bacterial hosts and this finding was contrasted in the more naïve *P. aeruginosa* derived phages (Tariq *et al*., 2015). The ability of a large subset of these phages to re-infect their originating host does not conform to the typical lambda model of phage infection. Fogg *et al* (2010) reviewed the lambda phage model, this model states that upon phage infection the infected bacterium is immune to further phage infection events including re-infection by the same phage. The re-infection model of phage infection shown in this chapter has also been shown by Allison *et al* (2003) when studying the *Escherichia coli* (*E. coli*) phage $24_{B.}$

In order to determine whether these phage infectivity and bacterial sensitivity profiles were due to the additive effect of multiple phage incorporation into the bacterial chromosome or due to a single predominant phage, we decided to generate plaque purified phages of the predominant phage species from 20 *P. aeruginosa* isolates. Polylysogeny has previously been seen in other *P.*

aeruginosa isolates such as LES which allowed the assumption that the *P. aeruginosa* isolates in this study were also polylysogens (Winstanley *et al.*, 2009). 5 *P. aeruginosa* isolates originating from each clinical aetiological subgroup were utilised in order to generate lysogens within a PAO1 backbone. These lysogens were compared against the non-lysogenic strains and differences in the infectivity profiles were observed.

The mixed phage *P. aeruginosa* isolates showed greater infectivity profiles compared to the plaque purified phages thus, enforcing the importance of polylysogeny in these clinical *P. aeruginosa* isolates. Polylysogeny is often overlooked within the chronic lung setting where only recent investigations are beginning to show its importance. However, James *et al* (2015) showed that there was a high prevalence of free temperate bacteriophages in CF sputum and so these phages may also influence interactions seen in the CF lung. These observations therefore, are novel but they also provide further indications of the future challenges which will need to be faced when treating phage infected *P. aeruginosa* isolates (James *et al.*, 2015).

Chapter 4 focused on a subset of the PAO1 lysogens generated in chapter 3 to allow for more detailed analysis to be undertaken to elucidate changes that occur in the bacterium's phenotype upon prophage inclusion. It has previously been shown that lysogenic strains of *E. coli* have an elevated growth rate in the lab, but this is one of the first instances where an elevation in growth rate has been shown in clinical *P. aeruginosa* lysogens (Edlin *et al.*, 1977, Edlin *et al.*, 1975, Lin *et al.*, 1977).

The lysogen which exhibited a reduction in bacterial growth was elucidated to harbour a prophage region originating from a < 10 BR phage. In chapter 3, it was identified that the *P. aeruginosa* isolates isolated from the < 10 BR patient cohort

had lower infection profiles and reduced bacterial sensitivity. It is possible to assume that this phage upon prophage formation may be integrating into a site on the chromosome that is deleterious to cell physiology and function. Another reason which may explain this deviation away from the trend may be that this phage has evolved in an environment where an elevation in the growth rate of the bacterial host is not essential thus, this phage has an alternative evolutionary strategy. When excluding this lysogen, it is possible to propose that the other phages maybe providing their bacterial hosts with a selective advantage. As an elevated growth rate may allow for the rapid establishment of a *P. aeruginosa* community within the lower lung. So it is possible to premise that this selective advantage enables *P. aeruginosa* to become the predominant bacterium in the CF/BR lung.

Research undertaken in chapter 6 describes that *P. aeruginosa* phages increase the metabolism of their host cells, so it is possible to hypothesis that this in turn drives the enhanced growth of the lysogenic strains. With increased rates of growth, there would be increased rates of host cell lysis. Metabolites released from these lysing cells into the surrounding environment could also potentially enhance the growth of the other cells residing in the community through alternate carbon turnover. These increased metabolites will also create an environment which is nutrient rich and so therefore, twitching motility may be elevated. Twitching may be elevated in a nutrient rich environment due to the large amount of metabolites available for the bacterium, the data presented here utilised LB media and this is a nutrient rich environment. So the data presented here is indicative of a nutrient rich environment.

Another advantage that the lysogens exhibited over the naïve PAO1 control was an elevation in tolerance to clinically relevant anti-pseudomonal antibiotics, again shown in chapter 4. Even though the antibiotics administered in this investigation

were at levels which are lower than the therapeutic dose of the drug, the pharmacokinetic profile of the CF/BR lung is poor and this provides clinicians with serious treatment problems. The thick mucus layer alongside damaged tissue could allow for resistance to build up in a small subset of the bacterial population because certain areas wouldn't be encountering antibiotics at optimal levels (Wright *et al*., 2013).

This resistance would therefore, increase the number of sensitive hosts for the phages thus, supporting their survival within the chronic lung. As antibiotic resistance is often chromosomally linked, resistance profiles within the bacterial community will change through selection. The increase in tolerance may have serious downstream consequences, as it could potentially indicate that suboptimal treatment of a bacterial infection will enhance the spread of phages around a particular niche due to the increased number of sensitive hosts which have not been eradicated through effective antibiotic treatment. The observation of polylysogeny occurring within these *P. aeruginosa* isolates is pertinent to mention here. To support this statement, this study has shown that a subset of *P. aeruginosa* isolates harboured multiple phages and these multiply infected isolates could potentially increase the spread of phage encoded resistance within a bacterial community. This has previously been seen and this led to the development of a *P. aeruginosa* strain (Liverpool Epidemic Strain/LES) which is resistant to entire classes of antibiotics (Ashish *et al*., 2012). It has also been shown that lysogenic strains have elevated growth rates therefore, these resistant bacterial cells would be able to successfully colonise a new niche thus, spreading antibiotic resistance.

The ability of CF lysogens to generate a small change upon normalisation for growth allows assumptions to be drawn that the CF phages can manipulate their host cells in such an effective way that the phages are in essence driving the

increase in antibiotic tolerance observed in chapter 4. In chapter 6, it was seen that the phage metagenomes for the adult CF phages had the highest incidence rate for metagenomes relating to 'xenobiotic degradation'. The metagenomic data supports the MIC profiles as they indicate that the CF phages can effectively clear antibiotics from an environment and so this would warrant the small change upon normalisation for growth. The BR phages generate a large change in tolerance upon normalisation for growth and it is proposed that this is because these phages can't manipulate their host cells as effectively as the CF phages.

Chapter 3 as mentioned previously showed that there were differences in the phage infectivity and bacterial sensitivity profiles. In order to determine whether there were any phenotypic differences in the *P. aeruginosa* isolates which potentially may drive these infectivity and sensitivity differences, chapter 4 looked to study the type IV pili. These appendages were studied as it has been described in previous studies that *P. aeruginosa* phages target the pili for adsorption and infection (Feary *et al.*, 1964, Bradley and Pitt, 1974, Bradley, 1973c, Olszak *et al.*, 2015, Mattick, 2002). Chapter 4 looked at molecular characterisation of these regions and the presence/absence of the type IV pili on the *P. aeruginosa* hosts was determined via polymerase chain reaction (PCR) amplification.

It was hypothesised that in accordance with previously published studies the BR isolates would be the most motile and the CF isolates would be the least motile, as motility has been perceived as an unnecessary and costly process for these bacterial isolates to retain (Mahenthiralingam *et al.*, 1994, Burke *et al.*, 1991). These data presented in chapter 4 supported the proposed hypothesis that the BR *P. aeruginosa* isolates would exhibit greater motility compared to the CF *P. aeruginosa* isolates (Mahenthiralingam *et al.*, 1994).

The paediatric CF phages were in fact less motile than the adult CF isolates when the distribution of motility was studied and this finding is in contrast to work shown by Kus *et al* (2004). It is pertinent to mention however, that the paediatric CF cohort used in this thesis has a low sample number (n = 10) and so the small cohort size may influence the results generated.

70 % of the *P. aeruginosa* isolates had type IV pili genes which were detectable via PCR amplification thus, potentially indicating the importance of these appendages for bacterial survival within the lung. However, these appendages may be detectable by PCR but the bacterium may not be motile due to other genetic alterations. When self-induced phage was added to the assays, there was not a consistent increase in motility or a consistent decrease. Phages are known to utilise the tRNA site (Ochman *et al*., 2000, Ritter *et al*., 1995, Inouye *et al*., 1991) as a receptor site, this lead to the proposal that in some instances phage incorporation can be beneficial for the bacterial isolates. However, in some instances it was observed that the addition of a phage decreased twitching. This would lead to the assumption that the phages may have incorporated into an essential motility gene thus, preventing bacterial movement. It is also possible that phage incorporation has increased the genetic size of the bacterium which has had a detrimental effect on the bacterium.

In order to confer as much depth to our knowledge about these clinical *P. aeruginosa* isolates, it was decided to focus a chapter of this thesis (chapter 5) on metabolomics. The aim was to determine whether there were specific metabolites which could perceivably drive the biological phenotypes observed in the previous chapters. A key point was to determine whether these phages brought any new metabolites to the system which would influence the phage biology results seen in the previous chapters.

Alongside metabolomics, it was possible to utilise metabolism studies to determine the impact on respiration which may be caused by phage infection and to see how this respiration is altered during pellicle formation. XTT results showed that phage addition to a pellicle culture could increase the respiration of the bacterial community residing within the pellicle. However, this observed increase was not always statistically significant. It was proposed that the time points used in these assays may have influenced the results seen as the initial harvesting step was post 24 hours of pellicle formation. The selection of this time point may have been too late in the developmental process in order to allow for the observations of significant metabolism alterations. In conclusion, we hypothesised that the mixed phage communities alter the metabolism of their *P. aeruginosa* host bacterium but the amount depends upon the bacterial isolate.

The pellicle cultures derived from CF *P. aeruginosa* isolates were more metabolically active on average compared to the pellicle cultures from the BR *P. aeruginosa* isolates. These findings are in accordance with the results shown in the previous chapters which indicate that the CF phages are the most adapted to co-evolve alongside *P. aeruginosa*. When observing the effect that phage addition had on pellicle formation, the addition of exogenous adult CF phage (24) was seen to cause the most significant elevation in respiratory metabolism. It may be assumed that this increase in respiration maintains both the phage and the bacteria within an environment that mostly consists of biofilm communities.

The metabolomic part of chapter 5 was focused upon the pellicle formation process utilising the lysogens generated and studied in chapter 4. Cell counts generated quantitative data to support the findings seen in the metabolomics study. An increase in cell number was determined through these counts, and this indicated that any differences observed in the metabolomic profiles were due to viable cells. As larger cell counts were detected as the pellicle formation process

continued, equal loading of the samples by dry weight excluded any bias in the analysis process.

Both PLS-DA and oPLS-DA plots were utilised to show the maximum variation present in the metabolomic data in this thesis. Both models showed that when using the *E. coli* metabolome database (ECMDB), the lysogens formed discrete groups irrespective of their clinical origin. This led to us concluding that the lysogens had different metabolite profiles which were linked to the developmental stage of the pellicle and that the individual phages were not driving the diverse profiles observed. However, when studying the individual time points in more depth, it was observed that the adult CF lysogen had a metabolite profile which showed the maximum variation compared to the naïve PAO1 profile. This observation was apparent in all the data sets shown on the models and this finding supports work that has been previously shown in this thesis relating to the complexity of the adult CF phages.

The previously described data has shown that the adult CF phages are the most adapted to co-evolve alongside *P. aeruginosa* and generate the greatest differences in phenotypic profiles compared to naïve controls. These metabolite profiles support these previous findings as they show that the metabolites generated by the adult CF lysogen are the most discrete compared to the control samples. The variation between the metabolite profiles was determined by the data point's location upon the x-axis of both plots.

The paediatric CF lysogen metabolite profile was also discrete compared to the control profile but the variation was reduced in relation to the adult CF lysogen. The metabolite profile which showed the least amount of variation was the < 10 BR lysogen. Again this finding is in support of previous data presented in this thesis.

In order to confer putative functionality to the metabolites generated, a VIP plot was generated from the PLS-DA plot and it showed 41 possible metabolites of interest. When the data was analysed using the ECMDB, no metabolites were detected which were significant, had a CV of ≤ 5 and a mass error of +/- 1. The lack of detection of a putative metabolite is perceived to be a result of there being no specific database for *P. aeruginosa* metabolomic analysis. The mass error value of 1 was selected for this analysis as it gave added support and strength to any metabolite identifications.

In order, to try and identify putative metabolites from the data presented in this chapter, the human metabolome database (HMDB) was utilised. However, no putative metabolites were detected using the previously described criteria but when the mass error was raised to +/- 2, there were 5 potential metabolites identified. Out of these metabolites identified, one appeared to warrant further investigation; Nitrofurazone (NTZ). However, in order to definitely confirm the presence of this metabolite MS/MS analysis would need to be undertaken because MS is not descriptive enough to allow for accurate metabolite identifications but MS/MS analysis was outside the remit of this thesis.

This thesis has thus far described differences in phage infectivity profiles, bacterial sensitivity, re-infection capabilities of phages, polylysogeny, lysogenic growth alterations, phage-derived antibiotic tolerance, motility, metabolism and metabolomics. The final chapter of this thesis focused on the metagenomic profiles of the phages in order to generate genetic data to describe the results previously observed. The adult CF phages throughout the previous chapters have been shown to be the most adapted to a particular environment and drive the greatest differences in phenotypic outcomes. The < 10 BR phages by contrast have been shown to the least adapted. In this final chapter, a metagenomic approach was used to link the phages genetic profile to a particular

patient subgroup. Changes in genes with annotated functions were specifically targeted as these genes may provide some clues into what phages encode and how these genes may aid bacterial survival within the chronic lung.

The metagenomics rapid annotations based on subsystem technology (MG-RAST) online portal was utilised in order to try and confer putative functionality to the shotgun sequenced phage lysates. The data presented in chapter 6 was also presented by Tariq and Everest *et al* (2015). This chapter along with the publication showed that bacterial chromosomal DNA can be hard to negate completely from a viral DNA preparation and these data have shown the importance of bioinformatic analysis to clear contaminating DNA prior to phage metagenomic analysis being undertaken (Tariq *et al.*, 2015).

These data presented in chapter 6 described that the adult CF metagenomes harboured the highest number of genes with putative functionalities when comparing the sequence data to kyoto encyclopaedia of genes and genomes (KEGG) pathway maps generated by MG-RAST. This finding therefore, provides genetic data to support the previous phage biology data and the previously proposed hypotheses, that the phages become more complex in their infection capabilities and generate a broader host range as CF progresses. This finding has a key clinical interest as it may be possible through future work to alter certain areas of the phages metagenome in order to control their spread and infectivity within a *P. aeruginosa* population.

When KEGG metagenomic profiles of CF and BR phages were analysed further, it was observed that the profiles altered according to the patient subgroup. The paediatric CF phage metagenomes showed reduced numbers of proposed identifications compared to the adult CF phage metagenomes thus, suggesting

that time may influences the genetic backbone of the phages which in turn drives the alterations previously observed in this thesis (Tariq *et al.*, 2015).

This alteration in the phage metagenomes was notable when studying the KEGG atlas section; glycan biosynthesis and metabolism (Tariq *et al.*, 2015). In this atlas map, the CF phages in particular the adult CF phages were seen to be the most enriched for these functions. Notably, the CF lung contains a large amount of mucins which are covered in glycans to which *P. aeruginosa* can adhere. Therefore, the CF phages may have evolved to aid glycan metabolism which would promote long term bacterial survival and the associated phage survival. This relationship is another example of the antagonistic co-evolution as proposed by Buckling and Rainey (2002).

This metagenomic chapter has potentially shown a 'snapshot' of phage evolution occurring within the lung. This evolutionary snapshot can be proposed because the metagenomes from the phages isolated from each clinical subgroup can be aligned next to each other and conclusions be drawn relating to their functionality profiles and the changes observed.

This research thesis has shown that temperate bacteriophages have a role in bacterial disease and the progression and severity of chronic respiratory diseases. This investigation has tried to answer the following research question "*are phages the new clinical challenge in the treatment of chronic respiratory diseases?"* I believe that through all the work undertaken that it is possible to answer the question. The answer would be yes, temperate bacteriophages do provide the bacteria with an added level of complexity which may have an effect on clinical treatments both now and in the future.

Future Work:

In order to further elucidate the relationships between bacteriophages and their bacterial host cells then a longitudinal study would be the ideal investigation to undertake and support the data presented in this thesis. A longitudinal study would allow the researcher to determine how the bacterial isolates evolve in order to prevent further phage infection whilst it would also allow a researcher to determine how phages have co-evolved to overcome these restrictions. It would be interesting to determine how the host range of the phages evolve and change throughout the course of the investigation whilst also observing 'real time' changes in the phages infectivity profiles.

A large majority of this work has been based upon the formation of prophages so further work elucidating the integration sites for these phage particles may also be of importance. The integrases involved in the formation of the prophage could be determined and this would give us some genetic information relating to the replication capabilities of these phages. It would also allow us to determine how the phages integrate into the bacterial chromosome and the effect that integration may impose on the bacterium's genetic profile as well as the phenotypic outcome. The *P. aeruginosa* isolates in this investigation have not been sequenced and this would be essential if this work was to progress further.

This thesis has shown how the field of phage evolution is constantly evolving and how there are numerous new studies which can be undertaken in order to elucidate the effects that phages impose upon their bacterial host cells.

Characterising the role and evolution of temperate bacteriophages in chronic respiratory infections including Cystic Fibrosis and Bronchiectasis

Francesca Louise Claire Everest

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Appendix 1: Clinical data relating to the samples involved in this investigation

CF sample ID	Strain Information	Adult / Paediatric	Date of isolation	Patient age upon isolation (years)	Phenotype
3	Same as 3	Adult	08.11.2010	32	Non mucoid
5	Manchester Epidemic Strain	Adult	09.11.2010	28	Non mucoid
6	Liverpool Epidemic Strain	Adult	09.11.2010	27	Non mucoid
16	Leeds Epidemic Strain	Adult	10.11.2010	25	Mucoid
23	Same as CF63	Adult	15.11.2010	18	Non mucoid
24	Same as CF183	Adult	15.11.2010	39	Non mucoid
28	Liverpool Epidemic Strain	Adult	18.11.2010	28	Non mucoid
30	Same as CF30	Adult	17.11.2010	23	Non mucoid
34	Liverpool Epidemic Strain	Adult	18.11.2010	38	Non mucoid
42	Same as CF121	Adult	22.11.2010	27	Non mucoid
44	Cluster E	Adult	24.11.2010	41	Non mucoid
52	Unique	Adult	26.11.2010	25	Mucoid
54	Unique	Adult	25.11.2010	17	Mucoid
55	Unique	Adult	25.11.2010	25	Non mucoid

57	Unique	Adult	25.11.2010	28	Non mucoid
60	Unique	Adult	26.11.2010	28	Non mucoid
63	Same as CF23	Adult	27.11.2010	29	Non mucoid
65	Clone C	Adult	27.11.2010	27	Non mucoid
67	Liverpool Epidemic Strain	Adult	29.11.2010	24	Non mucoid
72	Unique	Adult	30.11.2010	27	Non mucoid
74	Unique	Adult	01.12.2010	33	Mucoid
77	Same as nCFBR206	Adult	06.12.2010	33	Non mucoid
78	Clone C	Adult	06.12.2010	23	Mucoid
79	Unique	Adult	06.12.2010	33	Non mucoid
81	Manchester Epidemic Strain	Adult	08.12.2010	49	Mucoid
118	Non defined cluster 2	Adult	25.02.2011	31	Non mucoid
121	Same as CF42	Adult	25.02.2011	19	Non mucoid
125	Unique	Adult	16.02.2011	59	Non mucoid
126	Matches another local CF	Adult	16.02.2011	29	Non mucoid
127	Unique	Adult	18.02.2011	29	Non mucoid
136	Manchester Epidemic Strain	Adult	02.03.2011	24	Non mucoid
140	Same as CF208,	Adult	02.03.2011	36	Non mucoid

	CF214 and nCFBR53				
142	Manchester Epidemic Strain	Adult	02.03.2011	30	Non mucoid
145	Leeds Epidemic Strain	Adult	04.03.2011	44	Non mucoid
177	Midlands – 1 Epidemic Strain	Adult	26.04.2011	38	Non mucoid
183	Same as CF24	Adult	10.05.2011	39	Mucoid
211	Matches another local CF	Adult	23.09.2011	29	Non mucoid
47	Same as CF70	Paediatric	24.11.2010	16	Non mucoid
53	Unique	Paediatric	25.11.2010	4	Mucoid
69	Unique	Paediatric	30.11.2010	10	Non mucoid
70	Same as CF47	Paediatric	30.11.2010	17	Mucoid
124	Unique	Paediatric	22.02.2011	10	Non mucoid
165	Same as CF187	Paediatric	24.03.2011	1.8	Mucoid
187	Same as CF165	Paediatric	20.05.2011	2	Mucoid
208	Same as CF140, CF214 and nCFBR53	Paediatric	08.07.2011	15	Mucoid
213	Matches another local CF	Paediatric	16.09.2011	14	Non mucoid
214	Typing currently	Paediatric	11.11.2011	4	Non mucoid

BR sample ID	Strain Information	> 10 / < 10 BR	Date of isolation	Patient age upon isolation (years)	Phenotype
52	Unique	> 10	06.08.2008	56	Mucoid
53	Same as CF140, CF208 and CF214	> 10	06.08.2008	88	Mucoid
58	Unique	> 10	12.08.2008	44	Mucoid
136	Unique	> 10	13.10.2010	64	Mucoid
141	Unique	> 10	12.01.2011	71	Mucoid
143	Same as nCFBR123	> 10	12.01.2011	68	Non mucoid
144	Unique	> 10	02.02.2011	61	Mucoid
146	Unique	> 10	02.02.2011	72	Mucoid
153	Unique	> 10	15.03.2011	62	Mucoid
161	Unique	> 10	13.06.2011	65	Mucoid
177	Unique	> 10	14.07.2011	61	Non mucoid
178	Clone C	> 10	12.07.2011	56	Mucoid
181	Unique	> 10	13.07.2011	64	Non mucoid
195	Unique	> 10	14.07.2011	75	Non mucoid
197	Unique	> 10	20.07.2011	74	Non mucoid
199	Unique	> 10	20.07.2011	27	Non mucoid
200	Same as another nCFBR	> 10	21.07.2011	78	Non mucoid
201	Unique	> 10	20.07.2011	78	Mucoid

204	Cluster A	> 10	20.07.2011	70	Non mucoid
205	Same as another local CF	> 10	27.07.2011	61	Non mucoid Non mucoid
208	Unique	> 10	26.07.2011	61	
213	Unique	> 10	25.07.2011	64	Non mucoid
222	Same as another local CF	> 10	28.07.2011	33	Non mucoid
228	Unique	> 10	27.11.2011	60	Non mucoid
243	Clone C	> 10	30.08.2011	60	Non mucoid
244	Clone C	> 10	31.08.2011	76	Non mucoid
285	Not typed	> 10	23.09.2011	68	Mucoid
313	Not typed	> 10	17.20.2011	38	Mucoid
322	Not typed	> 10	27.10.2011	74	Non mucoid
326	Not typed	> 10	02.11.2011	73	Mucoid
59	Unique	< 10	12.08.2008	83	Mucoid
123	Same as nCFBR143	< 10	20.05.2010	74	Non mucoid
133	Unique	< 10	11.08.2010	82	Mucoid
150	Unique	< 10	04.03.2011	56	Mucoid
152	Unique	< 10	15.03.2011	85	Mucoid
193	Unique	< 10	21.07.2011	87	Mucoid
206	Same as CF77	< 10	27.07.2011	59	Mucoid
227	Unique	< 10	02.08.2011	81	Non mucoid

233	Not typed	< 10	04.08.2011	87	Mucoid
293	Not typed	< 10	27.09.2011	72	Non mucoid
299	Not typed	< 10	11.10.2011	80	Non mucoid
298	Not typed	< 10	12.10.2011	87	Non mucoid
319	Not typed	< 10	04.11.2011	60	Non mucoid
320	Not typed	< 10	02.11.2011	63	Non mucoid
327	Not typed	< 10	04.11.2011	81	Mucoid
331	Not typed	< 10	11.11.2011	78	Mucoid

Appendix 2: Clinical history of the BR patients

Strain ID	> 10 / < 10 BR	Clinical history
52	> 10	Ulcerative colitis
53	> 10	Idiopathic
58	> 10	Idiopathic
136	> 10	Rheumatoid arthritis associated
141	> 10	Idiopathic
143	> 10	Measles
144	> 10	Idiopathic
146	> 10	Rheumatoid arthritis associated
153	> 10	Post infectious
161	> 10	Pink's disease
177	> 10	Wegener's
178	> 10	Idiopathic
181	> 10	Specific antibody deficiency
195	> 10	Post infectious
197	> 10	Asthma
199	> 10	Primary ciliary dyskinesia
200	> 10	Idiopathic
201	> 10	Idiopathic
204	> 10	Colitis
205	> 10	Idiopathic
208	> 10	Idiopathic
213	> 10	Pinks
222	> 10	Idiopathic
228	> 10	Idiopathic
243	> 10	Post infectious (childhood pneumonia)
244	> 10	Post infectious (childhood pneumonia)
285	> 10	Idiopathic
313	> 10	Idiopathic
322	> 10	Idiopathic
326	> 10	Post infectious
59	< 10	Idiopathic

123	< 10	Asthma
133	< 10	Idiopathic
150	< 10	Post infectious
152	< 10	Idiopathic
193	< 10	Idiopathic
206	< 10	Idiopathic
227	< 10	COPD
233	< 10	Asthma
293	< 10	COPD
298	< 10	Idiopathic
299	< 10	Asthma
319	< 10	**Sjögren's**
320	< 10	Idiopathic
327	< 10	COPD
331	< 10	Idiopathic
332	< 10	Idiopathic

Appendix 3: Equipment utilised during this thesis including company information.

Equipment	Company	Company address
Plastic consumables	Greiner Bio	Stonehouse, UK
Static incubator	BINDER GmbH	Tuttlingen, Germany
Orbital incubator, Innova 44	Eppendorf	Stevenage, UK
Pipetes, BioPette PLUS	Labnet	Edison, NJ, USA
Spectrophotometer, Helicos	Spectronic Camspec Ltd	Leeds UK
Centrifuge, Sigma *3 – 18 KS*, rotor 11133 and 13104	Sigma Aldrich	Gillingham, UK
Plate Reader, Synergy HT	BioTek	Potton, UK
96 well cell culture plates	Sarstedt	Nümbrecht, Germany
Plate Reader software, Gen5 1.06	BioTek	Potton, UK
Desktop centrifuge, Sigma 1 -5	Paul Harris Scientific	Hyde, UK
Desktop centrifuge, MicroCentaur	DJB Labcare Limited	Newport Pagnell, UK
Vortex, Vortex Genie 2	Mo Bio	Cambridge, UK
NanoDrop, NanoDrop 1000 Spectrophotometer V3.7	Thermo Fisher Scientific Ltd	Cramlington, UK
PCR machine, C1000 Thermal Cycler	BioRad	Hemel Hempstead, UK
Power Pack, PowerPac Basic	BioRad	Hemel Hempstead, UK
Syngene, GeneSys software	Syngene Limited	Cambridge, UK
Freeze drier, Alpha 1 - 2	Scientific Laboratory Supplies	Hessle, UK
Balance, Acculab	Scientific Laboratory Supplies	Hessle, UK
Bandelin Sonorex, Sonicator	Bandelin Electronic	Berlin, Germany
Ultimate 3000 LC System	Thermo Fisher Scientific Ltd	Cramlington, UK
Q Exactive	Thermo Fisher Scientific Ltd	Cramlington, UK
Illumina MiSeq platform	Illumina	Saffron Walden, UK
Sorval centrifuge, Sorval RC 5 B Plus	Thermo Fisher Scientific Ltd	Cramlington, UK

Appendix 4: Lysogen growth rate raw data

Time (hrs)	PAO1	PAO1	PAO1	PAO1	CF24	CF24	CF24	CF24
0.00	0.109	0.112	0.11	0.112	0.145	0.156	0.148	0.155
0.30	0.215	0.227	0.144	0.135	0.239	0.25	0.263	0.289
1.00	0.128	0.113	0.135	0.135	0.161	0.165	0.169	0.172
1.30	0.231	0.347	0.108	0.1	0.225	0.251	0.242	0.235
2.00	0.21	0.268	0.116	0.116	0.215	0.265	0.245	0.243
2.30	0.213	0.246	0.136	0.148	0.246	0.272	0.245	0.251
3.00	0.179	0.259	0.179	0.179	0.273	0.287	0.284	0.312
3.30	0.192	0.266	0.192	0.198	0.349	0.37	0.368	0.405
4.30	0.278	0.344	0.29	0.308	0.471	0.518	0.454	0.603
5.30	0.375	0.439	0.381	0.423	0.521	0.57	0.575	0.628
6.30	0.468	0.728	0.453	0.542	0.646	0.731	0.738	0.813
7.30	0.606	0.728	0.621	0.65	0.813	0.825	0.817	0.932
8.30	0.714	0.783	0.73	0.772	0.855	0.906	0.888	1.063
9.30	0.838	0.86	0.767	0.887	0.957	0.974	0.959	1.122
18.00	1.136	1.39	1.076	1.025	1.344	1.462	1.414	1.609

Time (hrs)	PAO1	PAO1	PAO1	PAO1	CF70	CF70	CF70	CF70
0.00	0.109	0.112	0.11	0.112	0.147	0.143	0.151	0.148
0.30	0.215	0.227	0.144	0.135	0.264	0.242	0.236	0.24
1.00	0.128	0.113	0.135	0.135	0.174	0.167	0.165	0.165
1.30	0.231	0.347	0.108	0.1	0.242	0.219	0.207	0.227
2.00	0.21	0.268	0.116	0.116	0.224	0.202	0.191	0.225
2.30	0.213	0.246	0.136	0.148	0.269	0.232	0.191	0.235
3.00	0.179	0.259	0.179	0.179	0.326	0.284	0.227	0.212
3.30	0.192	0.266	0.192	0.198	0.419	0.357	0.272	0.257
4.30	0.278	0.344	0.29	0.308	0.583	0.48	0.369	0.255
5.30	0.375	0.439	0.381	0.423	0.622	0.505	0.427	0.229
6.30	0.468	0.728	0.453	0.542	0.733	0.621	0.57	0.218
7.30	0.606	0.728	0.621	0.65	0.873	0.797	0.717	0.285
8.30	0.714	0.783	0.73	0.772	0.857	0.842	0.882	0.39
9.30	0.838	0.86	0.767	0.887	0.842	0.865	1.02	0.496
18.00	1.136	1.39	1.076	1.025	0.785	0.916	1.651	1.317

Time (hrs)	PAO1	PAO1	PAO1	PAO1	BR123	BR123	BR123	BR123
0.00	0.109	0.112	0.11	0.112	0.117	0.134	0.119	0.123
0.30	0.215	0.227	0.144	0.135	0.17	0.192	0.185	0.187
1.00	0.128	0.113	0.135	0.135	0.157	0.171	0.178	0.184
1.30	0.231	0.347	0.108	0.1	0.085	0.093	0.096	0.119
2.00	0.21	0.268	0.116	0.116	0.087	0.099	0.095	0.136
2.30	0.213	0.246	0.136	0.148	0.089	0.104	0.101	0.154
3.00	0.179	0.259	0.179	0.179	0.091	0.108	0.103	0.189
3.30	0.192	0.266	0.192	0.198	0.095	0.115	0.102	0.207
4.30	0.278	0.344	0.29	0.308	0.112	0.163	0.107	0.306
5.30	0.375	0.439	0.381	0.423	0.126	0.258	0.119	0.381
6.30	0.468	0.728	0.453	0.542	0.148	0.384	0.148	0.423
7.30	0.606	0.728	0.621	0.65	0.194	0.644	0.203	0.484
8.30	0.714	0.783	0.73	0.772	0.233	0.719	0.274	0.56
9.30	0.838	0.86	0.767	0.887	0.303	0.746	0.39	0.591
18.00	1.136	1.39	1.076	1.025	0.574	1.255	0.428	0.612

Time (hrs)	PAO1	PAO1	PAO1	PAO1	BR136	BR136	BR136	BR136
0.00	0.109	0.112	0.11	0.112	0.125	0.125	0.131	0.137
0.30	0.215	0.227	0.144	0.135	0.181	0.181	0.191	0.183
1.00	0.128	0.113	0.135	0.135	0.174	0.179	0.213	0.214
1.30	0.231	0.347	0.108	0.1	0.151	0.193	0.214	0.22
2.00	0.21	0.268	0.116	0.116	0.15	0.166	0.192	0.251
2.30	0.213	0.246	0.136	0.148	0.214	0.243	0.231	0.26
3.00	0.179	0.259	0.179	0.179	0.228	0.252	0.269	0.293
3.30	0.192	0.266	0.192	0.198	0.25	0.253	0.267	0.323
4.30	0.278	0.344	0.29	0.308	0.475	0.388	0.427	0.654
5.30	0.375	0.439	0.381	0.423	0.586	0.593	0.554	0.915
6.30	0.468	0.728	0.453	0.542	0.711	0.642	0.615	1.039
7.30	0.606	0.728	0.621	0.65	0.854	0.788	0.716	1.169
8.30	0.714	0.783	0.73	0.772	0.916	0.786	0.768	1.306
9.30	0.838	0.86	0.767	0.887	0.972	0.78	0.746	1.314
18.00	1.136	1.39	1.076	1.025	1.213	0.769	0.689	1.614

Appendix 5: MIC raw data

Lysogen	+ Ceft	+ Ceft	+ Ceft	PAO1 + Ceft	PAO1 + Ceft	PAO1 + Ceft	Lysogen Growth	Lysogen Growth	Lysogen Growth	PAO1	PAO1	PAO1
CF24 (9 hrs)	0.524	0.581	0.759	0.193	0.239	0.265	1.184	1.379	1.300	0.786	0.888	0.988
CF24 (18 hrs)	0.336	0.582	0.529	0153	0.160	0.160	0.988	1.022	1.211	1.221	1.336	1.266
CF70 (9 hrs)	0.835	0.865	0.896	0.544	0.608	0.470	0.839	0.978	1.117	0.910	0.913	0.927
CF70 (18 hrs)	0.279	0.168	0.385	0.401	0.669	0.340	1.502	1.473	1.503	1.248	1.227	1.226
BR123 (9 hrs)	0.740	0.762	0.747	0.754	0.781	0.708	1.421	1.442	1.315	0.959	1.032	1.165
BR123 (18 hrs)	0.874	0.912	1.024	0.747	0.650	0.755	0.794	0.435	0.389	1.120	1.182	1.048
BR136 (9 hrs)	1.014	1.071	1.007	0.754	0.781	0.708	1.723	1.725	1.672	0.959	1.032	1.165
BR136 (18 hrs)	0.940	0.748	0.663	0.747	0.650	0.755	1.456	1.156	1.484	1.120	1.182	1.048

Lysogen	+ Colo	+ Colo	+ Colo	PAO1 + Colo	PAO1 + Colo	PAO1 + Colo	Lysogen Growth	Lysogen Growth	Lysogen Growth	PAO1	PAO1	PAO1
CF24 (9 hrs)	0.616	1.528	1.472	0.449	0.550	0.431	1.184	1.379	1.300	0.786	0.888	0.988
CF24 (18 hrs)	1.860	1.846	1.888	1.483	1.506	1.404	0.988	1.022	1.211	1.221	1.336	1.226
CF70 (9 hrs)	0.630	0.628	0.608	0.449	0.550	0.431	0.890	0.814	0.832	0.786	0.888	0.988
CF70 (18 hrs)	1.328	1.387	1.422	1.483	1.506	1.404	1.769	1.805	1.805	1.221	1.336	1.266
BR123 (9 hrs)	0.844	0.893	0.864	0.449	0.550	0.431	1.496	1.516	1.450	0.786	0.888	0.988
BR123 (18 hrs)	1.084	1.034	1.054	1.483	1.506	1.404	1.398	1.385	1.379	1.221	1.336	1.266
BR136 (9 hrs)	0.532	0.586	0.604	0.449	0.550	0.431	1.355	1.400	1.368	0.786	0.888	0.988
BR136 (18 hrs)	0.839	0.943	0.625	1.483	1.506	1.404	1.219	1.043	1.250	1.221	1.336	1.266

Lysogen	+ Mero	+ Mero	+ Mero	PAO1 + Mero	PAO1 + Mero	PAO1 + Mero	Lysogen Growth	Lysogen Growth	Lysogen Growth	PAO1	PAO1	PAO1
CF24 (9 hrs)	0.383	0.781	0.986	0.163	0.244	0.131	1.453	1.523	1.305	0.910	0.913	0.927
CF24 (18 hrs)	0.974	1.210	1.124	0.225	0.260	0.214	0.983	1.031	1.000	1.248	1.227	1.226
CF70 (9 hrs)	1.164	1.238	1.293	0.266	0.261	0.133	1.222	1.299	1.209	0.959	1.032	1.165
CF70 (18 hrs)	0.974	0.845	0.812	0.816	0.858	0.751	0.956	0.964	0.987	1.120	1.182	1.048
BR123 (9 hrs)	0.410	0.455	0.564	0.364	0.440	0.348	1.125	1.325	1.337	1.150	1.174	1.166
BR123 (18 hrs)	0.855	1.078	0.527	0.601	0.535	0.502	0.937	0.967	1.023	1.045	1.347	1.138
BR136 (9 hrs)	0.678	0.561	0.684	0.266	0.261	0.133	1.723	1.725	1.672	0.959	1.032	1.165
BR136 (18 hrs)	0.444	0.271	0.531	0.816	0.858	0.751	1.456	1.156	1.484	1.120	1.182	1.048

Lysogen	+ Pipp	+ Pipp	+ Pipp	PAO1 + Pipp	PAO1 + Pipp	PAO1 + Pipp	Lysogen Growth	Lysogen Growth	Lysogen Growth	PAO1	PAO1	PAO1
CF24 (9 hrs)	0.984	0.893	1.055	0.118	0.198	0.064	1.453	1.523	1.305	0.910	0.913	0.927
CF24 (18 hrs)	1.129	1.071	1.111	0.319	0.326	0.309	0.983	1.031	1.000	1.248	1.227	1.226
CF70 (9 hrs)	0.980	1.221	1.249	0.312	0.322	0.166	1.222	1.299	1.209	0.959	1.032	1.165
CF70 (18 hrs)	0.837	0.612	1.123	0.769	0.827	0.784	0.956	0.964	0.987	1.120	1.182	1.048
BR123 (9 hrs)	0.561	0.628	0.530	0.312	0.322	0.166	1.421	1.442	1.315	0.959	1.032	1.165
BR123 (18 hrs)	1.140	1.104	0.957	0.769	0.827	0.784	0.794	0.435	0.389	1.120	1.182	1.048
BR136 (9 hrs)	0.833	0.842	0.836	0.312	0.322	0.166	1.723	1.725	1.672	0.959	1.032	1.165
BR136 (18 hrs)	0.681	0.745	0.551	0.769	0.827	0.784	1.456	1.156	1.484	1.120	1.182	1.048

Appendix 6: Raw data for the twitching motility assays

	+ ϕ (mm)	+ ϕ (mm)	+ ϕ (mm)	Average (mm)	-ϕ (mm)	-ϕ (mm)	-ϕ (mm)	Average (mm)
PAO1	11	10	6	9	8	9	9	9
LESB58	7	9	6	7	4	9	8	7
CF3	5	9	6	7	7	7	5	6
CF5	6	0	6	4	6	7	8	7
CF6	12	19	13	15	18	21	20	20
CF16	6	7	5	6	6	5	6	6
CF23	24	16	19	20	23	19	20	21
CF24	5	6	6	6	4	5	8	6
CF28	5	3	6	5	6	4	7	6
CF30	5	0	5	3	4	6	0	3
CF34	11	14	13	13	17	18	19	18
CF42	8	10	11	10	23	11	20	18
CF44	4	4	4	4	8	6	8	7
CF47	13	12	14	13	22	17	15	18
CF52	4	2	0	2	4	5	4	4
CF53	4	7	5	5	5	3	3	4
CF54	4	4	6	5	2	5	5	4
CF55	17	18	23	19	14	19	14	16
CF57	16	14	15	15	17	16	19	17
CF60	21	22	11	18	10	19	18	16
CF63	16	14	12	14	22	19	24	22
CF65	9	16	13	13	21	24	20	22
CF67	17	20	12	16	24	21	24	23
CF69	9	15	18	14	18	24	16	19
CF70	5	4	2	4	4	4	5	4
CF72		4	5	5	7	6	7	7
CF74	15	15	13	14	15	13	12	13
CF77	28	21	22	24	39	36	22	32
CF78	13	10	12	12	23	17	22	21
CF79	12	10	16	13	12	13	13	13
CF81	5	6	4	5	6	5	4	5
CF118	4	5	5	5	6	5	9	7
CF121	11	15	11	12	15	18	16	16
CF124	20	29	28	24	25	28	28	27
CF125	5	4	3	4	5	7	8	7

CF126	3	4	4	4	0	7	9	5
CF127	4	4	0	3	4	7	4	5
CF136	2	4	0	2	3	6	4	4
CF140	3	5	3	4	5	3	5	4
CF142	4	4	4	4	3	3	5	4
CF145	3	6	4	4	6	6	6	6
CF165	1	2	6	3	3	5	4	4
CF177	1	4	4	3	4	3	3	3
CF183	6	5	0	4	9	6	9	8
CF187	3	5	4	4	3	2	3	3
CF208	5	5	5	5	6	0	7	4
CF211	0	4	5	3	5	4	2	4
CF213	0	5	4	3	6	6	7	6
CF214	6	4	6	5	5	8	8	7
BR52	4	0	4	3	5	7	0	4
BR53	12	13	12	12	20	16	16	17
BR58	15	10	14	13	11	17	21	16
BR59	15	11	18	15	17	20	23	20
BR123	10	8	0	6	0	6	6	4
BR133	9	10	8	9	2	6	0	3
BR136	7	6	7	7	13	9	7	10
BR141	10	7	16	11	13	12	19	15
BR143	12	10	12	11	25	20	21	22
BR144	11	8	10	10	12	7	0	6
BR146	5	3	5	4	5	6	4	5
BR150	22	22	12	19	23	26	25	25
BR152	20	20	23	21	35	38	30	34
BR153	17	17	16	17	23	30	25	26
BR161	11	18	14	14	23	24	22	23
BR177	12	13	13	13	16	16	12	15
BR178	9	14	13	12	15	9	13	12
BR181	9	15	10	11	20	22	17	20
BR193	16	24	19	20	16	22	15	18
BR195	19	19	20	19	13	20	23	19
BR197	17	14	25	19	15	32	22	23
BR199	11	11	11	11	20	16	19	18
BR200	22	14	12	16	23	21	19	21
BR201	14	19	13	15	20	15	9	15
BR204	20	20	20	20	17	17	16	17

BR204	7	6	0	4	9	5	0	5
BR205	5	6	5	5	5	5	4	5
BR208	5	8	0	4	8	8	7	8
BR213	9	7	8	8	9	8	10	9
BR222	4	7	0	4	14	11	9	11
BR227	0	8	7	5	5	0	6	4
BR228	9	7	7	8	11	11	11	11
BR233	5	10	6	7	9	7	6	7
BR243	6	10	6	7	4	9	12	8
BR244	8	8	7	8	12	7	10	10
BR285	7	7	7	7	10	9	10	10
BR293	6	6	6	6	6	9	9	8
BR298	0	8	9	6	8	12	0	7
BR299	4	5	2	4	8	10	0	6
BR313	7	6	7	7	10	6	8	8
BR319	4	8	0	4	10	11	0	7
BR320	7	4	3	5	10	0	10	7
BR322	6	3	6	5	7	8	8	8
BR326	5	0	7	4	6	8	4	6
BR327	5	4	3	4	4	4	6	5
BR331	2	3	6	4	8	8	7	8
BR332	0	5	4	3	5	4	7	5

Appendix 7: Raw data for the XTT Metabolism study

Strain	n = 1	n = 2	n = 3
CF24 (36 hrs)	0.200	0.239	0.156
+ CF24	0.500	0.409	0.372
+ CF70	0.397	0.349	0.348
+ BR123	0.469	0.495	0.416
+ BR136	0.367	0.485	0.235
CF24 (72 hrs)	0.221	0.229	0.271
+ CF24	0.377	0.359	0.374
+ CF70	0.304	0.270	0.185
+ BR123	0.456	0.520	0.095
+ BR136	0.260	0.219	0.244
CF24 (102 hrs)	0.113	0.380	0.476
+ CF24	0.601	0.245	
+ CF70	0.119	0.202	0.168
+ BR123	0.597	0.402	0.478
+ BR136	0.509	0.144	0.356
CF70 (36 hrs)	0.248	0.187	0.254
+ CF24	0.240	0.467	0.236
+ CF70	0.239	0.189	0.247
+ BR123	0.246	0.234	0.261
+ BR136	0.224	0.235	0.237
CF70 (72 hrs)	0.262	0.254	0.181
+ CF24	0.257	0.217	0.178
+ CF70	0.512	0.199	0.301
+ BR123	0.131	0.229	0.254
+ BR136	0.217	0.165	0.173
CF70 (102 hrs)	0.409	0.345	0.319
+ CF24	0.441	0.183	0.384
+ CF70	0.818	0.272	1.899
+ BR123	0.288	0.294	0.396
+ BR136	0.306	0.246	0.295

BR123 (36 hrs)	0.209	0.111	0.255
+ CF24	0.280	0.305	0.294
+ CF70	0.255	0.271	0.311
+ BR123	0.260	0.214	0.217
+ BR136	0.257	0.229	0.265
BR123 (72 hrs)	0.191	0.184	0.190
+ CF24	0.383	0.645	0.421
+ CF70	0.395	0.204	0.260
+ BR123	0.199	0.169	0.183
+ BR136	0.174	0.187	0.164
BR123 (102 hrs)	0.240	0.179	0.221
+ CF24	0.347	0.435	0.310
+ CF70	0.258	0.198	0.247
+ BR123	0.266	0.202	0.264
+ BR136	0.281	0.197	0.223
BR136 (36 hrs)	0.425	0.386	0.385
+ CF24	0.380	0.375	0.357
+ CF70	0.283	0.291	0.424
+ BR123	0.291	0.437	0.394
+ BR136	0.264	0.262	0.374
BR136 (72 hrs)	0.273	0.276	0.326
+ CF24	0.195	0.182	0.204
+ CF70	0.265	0.094	
+ BR123	0.445	0.278	0.495
+ BR136	0.454	0.562	0.480
BR136 (102 hrs)	0.752	0.308	0.615
+ CF24	0.347	0.435	0.310
+ CF70	0.234	0.373	0.527
+ BR123	0.280	0.524	0.461
+ BR136	0.481	0.377	0.371

Appendix 8: QUBIT 2.0 values for the mixed temperate phage lysates induced from clinical CF *P. aeruginosa* isolates.

CF sample ID	QUBIT 2.0 value (ng/µL)	Dilution required (Y/N)
3	0.800	Y
5	0.700	Y
6	0.400	N
16	0.264	N
23	0.886	Y
24	0.254	N
28	0.426	N
30	0.332	N
34	0.302	N
42	0.272	N
44	0.346	N
47	0.170	N
52	0.452	N
53	0.988	Y
54	0.710	Y
55	1.150	Y
57	0.370	N
60	0.226	N
63	1.170	Y
65	1.180	Y
67	0.266	N
69	0.330	N
70	0.856	Y
72	0.198	N
74	0.270	N
77	0.568	Y
78	1.490	Y
79	1.330	Y
81	1.120	Y
118	0.200	N
121	0.290	N
124	0.262	N
125	0.282	N
126	0.492	N
127	0.212	N

136	0.460	N
140	0.386	N
142	2.400	Y
145	0.314	N
165	3.100	Y
177	1.030	Y
183	1.920	Y
187	0.130	N
208	0.820	Y
211	N/A	N
213	0.378	N
214	0.306	N

Appendix 9: QUBIT 2.0 values for the mixed temperate phage lysates induced from clinical BR *P. aeruginosa* isolates.

BR sample ID	QUBIT 2.0 value (ng/µL)	Dilution required (Y/N)
52	0.864	Y
53	0.238	N
58	0.146	N
59	0.426	N
123	0.800	Y
133	N/A	N
136	N/A	N
141	N/A	N
143	0.198	N
144	0.132	N
146	N/A	N
150	0.134	N
152	0.588	Y
153	0.968	Y
161	0.164	N
177	0.284	N
178	0.344	N
181	N/A	N
193	0.418	N
195	0.170	N
197	0.160	N
199	0.234	N
200	0.104	N
201	0.128	N
204	1.680	Y
205	0.662	Y
206	N/A	N
208	0.178	N
213	1.320	Y
222	1.190	Y
227	0.296	N
228	0.130	N
233	0.228	N
243	0.186	N
244	0.116	N

285	0.824	Y
293	0.906	Y
298	1.020	Y
299	0.944	Y
313	1.140	Y
319	0.612	Y
320	1.090	Y
322	1.070	Y
326	0.960	Y
327	0.936	Y
331	0.934	Y
332	0.992	Y

Appendix 10: The raw data from MG RAST generated for all the samples which passed its internal quality control, 11/17 < 10 BR, 25/28 > 10 BR, 10/10 Pediatric CF and 36/37 Adult CF.

Clinical group	Sample ID	Number of Reads uploaded to MG-RAST
< 10 year BR	Phage50	135,229
	Phage52	479,192
	Phage68	698,674
	Phage72	496,093
	Phage73	618,612
	Phage74	439,489
	Phage76	23,898
	Phage77	278,743
	Phage80	862,415
	Phage86	404,422
	Phage91	383,572
> 10 year BR	Phage46	25,262
	Phage47	397,339
	Phage48	546,201
	Phage51	248,902
	Phage53	313,599
	Phage54	372,256
	Phage55	904,450
	Phage56	258,012
	Phage59	368,431
	Phage60	39,831
	Phage61	382,264
	Phage62	274,339
	Phage63	362,896
	Phage64	326,612
	Phage65	1,018,124
	Phage69	415,920
	Phage75	97,351
	Phage78	708,098
	Phage79	442,845
	Phage85	236,330
	Phage87	17,953

	Phage88	540,788
	Phage89	1,115,698
	Phage92	594,975
	Phage93	62,894
Paediatric CF	Phage13	606,526
	Phage14	232,852
	Phage22	26,409
	Phage23	17,862
	Phage32	308,499
	Phage40	710,926
	Phage43	1,020,308
	Phage44	337,907
	Phage45	24,446
	Phage83	15,484
Adult CF	Phage1	336,053
	Phage2	10,860
	Phage3	1,112,907
	Phage4	46,152
	Phage5	335,623
	Phage6	453,949
	Phage7	307,972
	Phage8	649,773
	Phage9	809,956
	Phage10	25,305
	Phage12	57,446
	Phage15	371,506
	Phage16	806,898
	Phage17	343,106
	Phage18	983,127
	Phage19	1,035,849
	Phage20	238,769
	Phage21	1,092,624
	Phage24	19,468
	Phage25	129,064
	Phage26	93,154
	Phage27	90,725
	Phage28	978,403
	Phage29	256,542
	Phage30	56, 252

	Phage31	397,022
	Phage33	434,882
	Phage34	186,544
	Phage35	493,891
	Phage36	355,818
	Phage37	252,476
	Phage38	16,271
	Phage39	653,032
	Phage41	442,560
	Phage42	685,801
	Phage84	44,228

Appendix 11: KEGG putative functionality kits

GLYCAN BIOSYNTHESIS AND METABOLISM	KEGG Identification	Putative function
Paediatric CF	Lipopolysaccharide biosynthesis	Cell wall function
< 10 BR	Glycosphingolipid biosynthesis	Involved in inflammation in chronic lung diseases
Adult CF	Other types of O-glycan biosynthesis	Mucin binding domain – phage may encode to aid its attachment (serine/threonine base)
	Glycosphingolipid biosynthesis - ganglio series	Involved in inflammation in CF
	Lipopolysaccharide biosynthesis	Synthesis of lipopolysaccharides
> 10 BR	Sphingolipid metabolism	Involved in inflammation in chronic respiratory diseases
	Glycosphingolipid biosynthesis - lacto and neolacto series	Involved in inflammation in chronic respiratory diseases
	Other types of O-glycan biosynthesis	Mucin binding domain – phage may encode to aid its attachment (serine/threonine base)
	Various types of N-glycan biosynthesis	Involved in correct protein folding (aspartate base)
	Galactose metabolism	Energy – sugar
	Other glycan degradation	Degradation of linked monosaccharides
	Glycosaminoglycan degradation	Long unbranched polysaccharides made of repeating disaccharide units
	Glycosphingolipid biosynthesis - ganglio series	Involved in inflammation in chronic respiratory diseases
	Lysosome	Break down of biomolecules including proteins, amino

		acid, lipids and CHO – found in animal cells
LIPID METABOLISM	**KEGG Identification**	**Putative function**
Paediatric CF	Sphingolipid metabolism	Involved in inflammation in CF – possible drug target
	Other types of O-glycan biosynthesis	Mucin binding domain – phage may encode to aid its attachment
	Glycosphingolipid biosynthesis - ganglio series	Involved in inflammation in CF
	Fatty acid degradation	Degradation of fatty acids
	alpha-Linolenic acid metabolism	Alter the Fatty Acids in CF – have an effect on potential CF diet management
	Fatty acid metabolism	Fatty Acid metabolism
	Valine, leucine and isoleucine degradation	These are previously seen to be elevated in the CF lung
	Geraniol degradation	Geraniol (bacterial nutrient source)
	Benzoate degradation	Utilize alternative Carbon sources – longevity
	Ethylbenzene degradation	Plant originating metabolite
	Glycerolipid metabolism	Involved in inflammation in CF
	Glycerophospholipid metabolism	Involved in inflammation in CF
	Ether lipid metabolism	One or more Carbons on glycerol is bound to an alkyl chain via an ether linkage rather than the usual ester linkage
	Fc gamma R-mediated phagocytosis	Phagocytosis/Engulfment of cells mediated by the Fc domain on certain cellular proteins
	Fat digestion and	Degradation and absorption

	absorption	of fats
	Fatty acid biosynthesis	Fatty acid biosynthesis
	AMPK signaling pathway	Cellular homeostasis – phage utilizes to enhance its survival
	Insulin signaling pathway	Insulin
	Lysine degradation	Lysine degrades DNA
	Phenylalanine metabolism	Amino acid metabolism
	Tryptophan metabolism	Amino acid metabolism
	beta - Alanine metabolism	Amino acid metabolism
	Aminobenzoate degradation	Aminobenzonate has been trialed as a drug previously
	Proponate metabolism	Precursor to propanoic acid
	Butanoate metabolism	Precursor to butanoic acid
	Limonene and pinene degradation	Bronchitis drug – phage can breakdown
	Caprolactam degradation	Natural product that is the basis of some drugs – phage aid breakdown?
	alpha-Linolenic acid metabolism	Essential Fatty Acid
	Biosynthesis of unsaturated fatty acids	Unsaturated Fatty Acid synthesis
	PPAR signaling pathway	Link to obesity/insulin etc.
	cAMP signaling pathway	Energy generation
	Peroxisome	Link to obesity/insulin etc.
	Steroid biosynthesis	Steroid generation
< 10 BR	Ether lipid metabolism	One or more carbons on glycerol is bound to an alkyl chain via an ether linkage rather than the usual ester linkage
	Fatty acid biosynthesis	Biosynthesis of fatty acids

	Glycerophospholipid metabolism	Involved in inflammation in chronic lung diseases
	Fatty acid biosynthesis, initiation	Initiation of fatty acid biosynthesis
	Fatty acid biosynthesis, elongation	Elongation stage of fatty acid biosynthesis
	Fatty acid degradation	Degradation of fatty acids
Adult CF	Sphingolipid metabolism	Involved in inflammation in CF
	Taurine and hypotaurine metabolism	Taurine is found in a large proportion of animal tissues, hypotaurine is an intermediate in this process
	Cyanoamino acid metabolism	Amino acid metabolism – addition of a nitrile group to the front of the amino acid
	Glutathione metabolism	Antioxidant that protects cells from the damage caused by ROS (Reactive Oxygen Species)
	Arachidonic acid metabolism	Unsaturated fatty acid
	Fatty acid degradation	Degradation of fatty acids
	alpha-Linolenic acid metabolism	Alter the fatty acid in CF – have an effect on potential CF diet management
	Fatty acid metabolism	Self-explanatory
	Glycerolipid metabolism	Lipid metabolism
	Glycerophospholipid metabolism	Involved in inflammation in CF
	Fatty acid biosynthesis	Biosynthesis of fatty acids
	AMPK signaling pathway	Cellular homeostasis – phage utilizes to enhance its survival
	Insulin signaling pathway	Insulin
	Steroid hormone biosynthesis	Synthesis of steroid hormone's

	Metabolism of xenobiotics by cytochrome P450	Cytochrome P450's are key in xenobiotic degradation
	Chemical carcinogenesis	Chemicals that are capable of causing cancer
	Valine, leucine and isoleucine degradation	These are previously seen to be elevated in the CF lung
	Geraniol degradation	Geraniol (bacterial nutrient source) – phage utilize for energy
	Lysine degradation	Lysine degrades DNA
	Phenylalanine metabolism	Amino acid metabolism
	Benzoate degradation	Utilize alternative Carbon sources – longevity
	Tryptophan metabolism	Amino acid metabolism
	beta-Alanine metabolism	Amino acid metabolism
	Aminobenzoate degradation	Aminobenzonate has been trialed as a drug previously
	Propanoate metabolism	Precursor to propanoic acid
	Butanoate metabolism	Precursor to butanoic acid
	Limonene and pinene degradation	Drug degradation – bronchitis drug
	Caprolactam degradation	Natural product that is the basis of some drugs
	Synthesis and degradation of ketone bodies	Water soluble molecules produced in times of starvation, produced from Acetyl CoA
	Carbon fixation pathways in Prokaryotes	Fixation of carbon in Prokaryotes
	Terpenoid backbone biosynthesis	Secondary metabolite biosynthesis
	Ethylbenzene degradation	Utilize alternative Carbon sources – longevity

	Ether lipid metabolism	One or more Carbons on glycerol is bound to an alkyl chain via an ether linkage rather than the usual ester linkage
> 10 BR	Fatty acid degradation	Degradation of fatty acids
	alpha-Linolenic acid metabolism	Alter the fatty acids in CF – have an effect on potential CF diet management
	Glycerophospholipid metabolism	Involved in inflammation in chronic lung diseases
	Ether lipid metabolism	One or more Carbons on glycerol is bound to an alkyl chain via an ether linkage rather than the usual ester linkage
	Ras signaling pathway	Control of cellular signaling, normally involved in pathways that are involved in cell growth, differentiation and survival
	cAMP signaling pathway	Energy generation
	Endocytosis	Process where the cell engulfs products such as proteins, uses energy
	Fc gamma R-mediated phagocytosis	Phagocytosis/Engulfment of cells mediated by the Fc domain on certain cellular proteins
	Glutamatergic synapse	Post synaptic synapse – NMDA type
	GnRH signaling pathway	Gonadotropin-releasing hormone generated (benzene ring product)
	Sphingolipid metabolism	Involved in inflammation in chronic respiratory diseases
	Phosphonate and phosphinate	Degradable organic Carbon products which can be used

	metabolism	for pest control – benzene ring containing
	Fatty acid biosynthesis	Biosynthesis of fatty acid's
	Fatty acid metabolism	Metabolism of fatty acid's
	AMPK signaling pathway	Cellular homeostasis – phage utilizes in order to enhance its survival
	Insulin signaling pathway	Insulin productio
	Valine, leucine and isoleucine degradation	These are seen to be elevated in the CF lung
	Geraniol degradation	Geraniol (bacterial nutrient source)
	Lysine degradation	Lysine degrades DNA
	Phenylalanine metabolism	Amino acid metabolism
	Benzoate degradation	Utilize alternative Carbon sources – longevity Phage encoded
	Tryptophan metabolism	Amino acid metabolism
	beta-Alanine metabolism	Amino acid metabolism
	Aminobenzoate degradation	Aminobenzoate has been trialed as a drug previously
	Propanoate metabolism	Precursor to propanoic acid
	Butanoate metabolism	Precursor to butanoic acid
	Limonene and pinene degradation	Bronchitis drug – phage can breakdown
	Caprolactam degradation	Natural product that is the basis of some drugs – phage aid breakdown
	Lipopolysaccharide biosynthesis	Cell wall
	Steroid hormone biosynthesis	Synthesis of steroid hormone's
	Metabolism of xenobiotics by cytochrome P45	Using cytochrome P45 to generate xenobiotics

	Chemical carcinogenesis	Chemicals that are capable of causing cancer
Metabolism of Terpenoids and Polyketides (secondary metabolites naturally produced)	**KEGG Identification**	**Putative function**
Paediatric CF	Bisphenol degradation	Common chemical exposed to in daily life
	Polycyclic aromatic hydrocarbon degradation	Break down of compounds containing these rings
	Aminobenzoate degradation	Drug degradation
	Limonene and pinene degradation	Drug degradation – bronchitis drug
	Stilbenoid, diarylheptanoid and gingerol biosynthesis	Plant related drug
< 10 BR	Zeatin biosynthesis	Benzene ring plant hormone
	Diterpenoid biosynthesis	Benzene ring plant hormone
	Biosynthesis of secondary metabolites	Generation of secondary metabolites
Adult CF	Brassinosteroid biosynthesis	Plant hormone with a potential role in agricultural processes
	Terpenoid backbone biosynthesis	Secondary metabolite biosynthesis
> 10 BR	Diterpenoid biosynthesis	Potential use for antibacterial functions, plant based metabolite
	Brassinosteroid biosynthesis	Plant hormone with a potential role in agricultural processes
CARBOHYDRATE METABOLISM	**KEGG Identification**	**Putative function**
Paediatric CF	Galactose metabolism	Energy - sugar
	Other glycan	Chemicals that are capable

	degradation	of causing cancer
	Glycosaminoglycan degradation	Long unbranched polysaccharides made of repeating disaccharide units
	Sphingolipid metabolism	Involved in inflammation in CF
	Glycosphingolipid biosynthesis - ganglio series	Involved in inflammation in CF
	Lysosome	Break down of biomolecules including proteins, amino acid, lipids and CHO – found in animal cells
	Phosphotransferase system (PTS)	Sugar transport system
	Pentose and glucuronate interconversions	Transferring sugar groups
	Fructose and mannose metabolism	Amino acid metabolism
	Glycerolipid metabolism	Plant originating metabolite
	Bisphenol degradation	Common chemical exposed to in daily life
	Linoleic acid metabolism	Unsaturated fatty acid
	Chloroalkane and chloroalkene degradation	Degrading chloro derivative alkene's and alkane's
	Butanoate metabolism	Precursor to butanoic acid
	Steroid hormone biosynthesis	Biosynthesis of steroid hormone's
	Metabolism of xenobiotics by cytochrome P450	Cytochrome P450's are key in xenobiotic degradation
	Chemical carcinogenesis	Chemicals that are capable of causing cancer
	Methane metabolism	Metabolism of methane
	Carbon metabolism	Metabolism of carbon

	Carbon fixation in photosynthetic organisms	Transfer of inorganic Carbon to organic Carbon
	Glyoxylate and dicarboxylate metabolism	Reactions involving glyoxylate and dicarboxylate
	Glycine, serine and threonine metabolism	Amino acid metabolism
	Alanine, aspartate and glutamate metabolism	Amino acid metabolism
	Peroxisome	Break down of long chain fatty acids by beta oxidation
	Citrate cycle (TCA cycle)	Energy generation via the oxidization of acetate
	Glutathione metabolism	Antioxidant that protects cells from the damage caused by ROS (Reactive Oxygen Species)
	2-Oxocarboxylic acid metabolism	The most elementary set of metabolites -pyruvate (2-oxopropanoate), 2-oxobutanoate, oxaloacetate (2-oxosuccinate) and 2-oxoglutarate
	Biosynthesis of amino acids	Amino acid biosynthesis
	Glycolysis / Gluconeogenesis	Converts glucose to pyruvate/Generation of glucose from non-Carbon sources
	Fatty acid degradation	Degradation of fatty acid's
	Valine, leucine and isoleucine degradation	These are previously seen to be elevated in the CF lung
	Lysine degradation	Lysine degrades DNA
	Arginine and proline metabolism	Amino acid metabolism
	Histidine metabolism	Amino acid metabolism
	Tryptophan metabolism	Amino acid metabolism

	beta-Alanine metabolism	Amino acid metabolism
	Pyruvate metabolism	Glycolysis end product
	Limonene and pinene degradation	Bronchitis drug – phage can breakdown
	Propanoate metabolism	Precursor to propanoic acid
	GABAergic synapse	Neurotransmitter in CNS (Central Nervous System)
	Pentose phosphate pathway	Parallel to glycolysis and generates NAPDH and pentose
	Ascorbate and aldarate metabolism	Ascorbate – Vitamin C by-product Aldarate – acid generated from oxidation of the terminal groups on aldose
	Caprolactam degradation	Natural product that is the basis of some drugs
	Cyanoamino acid metabolism	Amino acid metabolism – addition of a nitrile group to the front of the amino acid
	Tryptophan metabolism	Amino acid metabolism
	Sulphur metabolism	Metabolism of sulphur
	Cysteine and methionine metabolism	Metabolism of cysteine and methionine
	Taurine and hypotaurine metabolism	Taurine is found in a large proportion of animal tissues, hypotaurine is an intermediate in this process
< 10 BR	Fructose and mannose metabolism	Amino acid metabolism
	Glycolysis	Converts glucose to pyruvate
	Nucleotide sugar biosynthesis	Biosynthesis of nucleotide sugars
	Streptomycin biosynthesis	Antimicrobial – protein synthesis inhibitor - Aminoglycoside

	Polyketide sugar unit biosynthesis	Secondary metabolites produced by most living organisms – contain a benzene ring
	Lipopolysaccharide biosynthesis	Cell wall
	Gluconeogenesis	Generation of glucose from non-Carbon sources
	Pentose phosphate pathway	Parallel to glycolysis and generates NAPDH and pentose
	Glyoxylate and dicarboxylate metabolism	Reactions involving glyoxylate and dicarboxylate
Adult CF	Galactose metabolism	Energy – sugar
	Other glycan degradation	Degradation of glycan's
	Glycosaminoglycan degradation	Long unbranched polysaccharides made of repeating disaccharide units
	Sphingolipid metabolism	Involved in inflammation CF
	Glycosphingolipid biosynthesis - ganglio series	Involved in inflammation CF
	Lysosome	Break down of biomolecules including proteins, amino acid, lipids and CHO – found in animal cells
	Starch and sucrose metabolism	Energy generation
	Fructose and mannose metabolism	Amino acid metabolism
	Glycolysis / Gluconeogenesis	Converts glucose to pyruvate/Generation of glucose from non-Carbon sources
	Amino sugar and nucleotide sugar	Generation of components of nucleic acids

	metabolism	
	Streptomycin biosynthesis	Antimicrobial – protein synthesis inhibitor - Aminoglycoside
	Butirosin and neomycin biosynthesis	Aminoglycosides – drugs that originate from bacterial species
	Carbon metabolism	Metabolism of carbon
	HIF-1 signaling pathway	Cellular signaling - participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival
	Carbohydrate digestion and absorption	Digestion and absorption of carbohydrate's
	Pyruvate metabolism	End product of glycolysis
	Methane metabolism	Methane metabolism
	Carbon fixation in photosynthetic organisms	Fixing carbon in photosynthetic organisms
	Carbon fixation pathways in Prokaryotes	Fixation of carbon in Prokaryotes
	Glyoxylate and dicarboxylate metabolism	Reactions involving glyoxylate and dicarboxylate
	Pentose phosphate pathway	Parallel to glycolysis and generates NAPDH and pentose
	Biosynthesis of ansamycins	Secondary metabolite – benzene ring product Antimicrobial activity
	Biosynthesis of amino acids	Synthesis of amino acids
	Glycine, serine and threonine metabolism	Amino acid metabolism
	Glycerolipid metabolism	Lipid metabolism
	Bisphenol degradation	Common chemical exposed

		to in daily life
	Linoleic acid metabolism	Unsaturated fatty acid
	Chloroalkane and chloroalkene degradation	Degrading chloro derivative alkene's and alkane's
	Butanoate metabolism	Precursor to butanoic acid
	Pentose and glucuronate interconversions	Transferring sugar groups
	Ascorbate and aldarate metabolism	Ascorbate – Vitamin C by-product Aldarate – acid generated from oxidation of terminal groups on aldose
	Fatty acid degradation	Degradation of fatty acid's
	Valine, leucine and isoleucine degradation	These are seen to be elevated in the CF lung
	Lysine degradation	Lysine degrades DNA
	Arginine and proline metabolism	Amino acid metabolism
	Histidine metabolism	Amino acid metabolism
	Tryptophan metabolism	Amino acid metabolism
	beta-Alanine metabolism	Amino acid metabolism
	Glycerolipid metabolism	Lipid metabolism
	Limonene and pinene degradation	Bronchitis drug – phage can breakdown
	Citrate cycle (TCA cycle)	Energy generation via the oxidation of acetate
	Benzoate degradation	Utilize alternative Carbon sources – longevity
	Bisphenol degradation	Common chemical exposed to in daily life
	Naphthalene degradation	Double benzene ring product – used in mothballs
	Butanoate metabolism	Precursor to butanoic acid
	2-Oxocarboxylic acid	The most elementary set of

	metabolism	metabolites – pyruvate, oxaloacetate and 2 – oxoglutarate
	C5-Branched dibasic acid metabolism	Involved in acid/base reactions
> 10 BR	Mannosyl phosphate transferase	Important in human metabolism – energy
	Galactose metabolism	Energy metabolism
	Other glycan degradation	Glycan degradation
	Glycosaminoglycan degradation	Long unbranched polysaccharides made of repeating disaccharide units
	Sphingolipid metabolism	Involved in inflammation in chronic respiratory diseases
	Glycosphingolipid biosynthesis - ganglio series	Involved in inflammation in chronic respiratory diseases
	Lysosome	Break down of biomolecules including proteins, amino acid, lipids and carbohydrates – found in animal cells
	Pentose phosphate pathway	Parallel to glycolysis and generates NEPDH and pentose
	Glutathione metabolism	Antioxidant that protects cells from the damage caused by ROS (Reactive Oxygen Species)
	Carbon metabolism	Carbon metabolism
	Central Carbon metabolism in cancer	Carbon metabolism in cancer
	Pentose and glucuronate interconversions	Transferring sugar groups
	Ascorbate and aldarate metabolism	Ascorbate – Vitamin C by-product Aldarate – acid generated

		from oxidation of terminal groups on aldose
	Starch and sucrose metabolism	Sugar metabolism
	Amino sugar and nucleotide sugar metabolism	Generation of components of nucleic acids
	Glycolysis / Gluconeogenesis	Converts glucose to pyruvate/Generation of glucose from non-Carbon sources
	Fructose and mannose metabolism	Amino acid metabolism
	Galactose metabolism	Energy – sugar
	Streptomycin biosynthesis	Antimicrobial – protein synthesis inhibitor - Aminoglycoside
	Butirosin and neomycin biosynthesis	Aminoglycosides – drugs that originate from bacterial species
	HIF-1 signaling pathway	Cellular signaling - participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival
	Insulin signaling pathway	Insulin production
	Type II diabetes mellitus	Related to insulin
	Carbohydrate digestion and absorption	Digestion and absorption of carbohydrates
	Alanine, aspartate and glutamate metabolism	Amino acid metabolism
	Cysteine and methionine metabolism	Amino acid metabolism
	Sulphur metabolism	Metabolism of sulphur
	Carbon metabolism	Metabolism of carbon
	Biosynthesis of amino acids	Biosynthesis of amino acids

	Histidine metabolism	Amino acid metabolism
	Fatty acid degradation	Degradation of fatty acids
	Valine, leucine and isoleucine degradation	These are seen to be elevated in the CF lung
	Lysine degradation	Lysine degrades DNA
	Arginine and proline metabolism	Amino acid metabolism
	Histidine metabolism	Amino acid metabolism
	Tryptophan metabolism	Amino acid metabolism
	beta-Alanine metabolism	Amino acid metabolism
	Glycerolipid metabolism	Lipid metabolism
	Pyruvate metabolism	End product of glycolysis
	Chloroalkane and chloroalkene degradation	Degrading chloro derivative alkene's and alkane's
	Limonene and pinene degradation	Bronchitis drug – phage can breakdown
	aldehyde dehydrogenase	Oxidise aldehyde's
	Glyoxylate and dicarboxylate metabolism	Reactions involving glyoxylate and dicarboxylate
	Citrate cycle (TCA cycle)	Energy generation via the oxidation of acetate
	Carbon fixation in photosynthetic organisms	Fixation of carbon in photosynthetic organisms
	Methane metabolism	Metabolism of methane
	Carbon fixation pathways in prokaryotes	Fixation of carbon in Prokaryotes
	Benzoate degradation	Utilize alternative Carbon sources – longevity
	Bisphenol degradation	Common chemical exposed to in daily life
	Naphthalene degradation	Double benzene ring product – used in mothballs

	Butanoate metabolism	Precursor to butanoic acid
NUCLEOTIDE METABOLISM	**KEGG Identification**	**Putative function**
Paediatric CF	Pyrimidine metabolism	Nucleotide protein – Cytosine/Thymine/Uracil
	beta-Alanine metabolism	Amino acid metabolism
	Pantothenate and CoA biosynthesis	Pantothenate is needed in animals to combine with CoA CoA is involved in fatty acid metabolism and the TCA cycle
	Drug metabolism - other enzymes	Drug metabolism
	Purine metabolism	Nucleotide protein – Adenine/Guanine
	DNA replication	Replication of DNA
	Base excision repair	Involved in Nucleotide Excision Repair
	Nucleotide excision repair	Removal of large damaged amino acids before mutations form
	Homologous recombination	Repairs DSB (Double Strand Breaks)
	Riboflavin metabolism	Vitamin B2, important cofactor in FAD which are essential in pathways such as - fatty acid metabolism, citrate cycle and electron transport chain
	Nicotinate and nicotinamide metabolism	Nicotinamide (B vitamin) is the amide of nicotinic acid which is an essential human nutrient
	Tyrosine metabolism	Amino acid metabolism
	Biotin metabolism	B vitamin, involved in Gluconeogenesis
< 10 BR	Pyrimidine degradation	Degrading nucleotide

		proteins – Cytosine/Thymine/Uracil
	Purine metabolism	Nucleotide protein metabolism – Adenine/Guanine
	Pyrimidine metabolism	Metabolizing nucleotide proteins – Cytosine/Thymine/Uracil
	DNA replication	Replication of DNA
	Base excision repair	Involved in Nucleotide Excision Repair
	Nucleotide excision repair	Removal of large damaged amino acids before mutations form
	Homologous recombination	Repairs DSB (Double Strand Breaks)
	Tyrosine metabolism	Amino acid metabolism
	Tryptophan biosynthesis	Amino acid metabolism
	Tyrosine degradation	Amino acid degradation
	Phenylalanine metabolism	Amino acid metabolism
	Glycine, serine and threonine metabolism	Amino acid metabolism
	Cysteine and methionine metabolism	Amino acid metabolism
	Arginine and proline metabolism	Amino acid metabolism
	Glutathione metabolism	Antioxidant that protects cells from the damage caused by ROS (Reactive Oxygen Species)
	Alanine, aspartate and glutamate metabolism	Amino acid metabolism
	Nitrogen metabolism	Part of the nitrogen cycle – energy for the cell
Adult CF	Tyrosine metabolism	Amino acid metabolism
	Styrene degradation	Benzene ring structure

	Alanine, aspartate and glutamate metabolism	Amino acid metabolism
	Arginine and proline metabolism	Amino acid metabolism
	Glyoxylate and dicarboxylate metabolism	Reactions involving glyoxylate and dicarboxylate
	Nitrogen metabolism	Nitrogen metabolism
	Biosynthesis of amino acids	Amino acid biosynthesis
	Two-component system	Response to the environment, allows adaptation to changing environments
	Glutamatergic synapse	Post synaptic synapse – NMDA type
	GABAergic synapse	Neurotransmitter in CNS (Central Nervous System)
	Pyrimidine metabolism	Amino acid metabolism
	beta-Alanine metabolism	Amino acid metabolism
	Pantothenate and CoA biosynthesis	Pantothenate is needed in animals to combine with CoA CoA is involved in fatty acid metabolism and the TCA cycle
	Drug metabolism - other enzymes	Drug metabolism
	Biotin metabolism	B vitamin, involved in Gluconeogenesis
	Carbapenem biosynthesis	Beta lactam antibiotic
	2-Oxocarboxylic acid metabolism	The most elementary set of metabolites – pyruvate, oxaloacetate and 2 – oxoglutarate
	Glutathione metabolism	Antioxidant that protects cells from the damage

		caused by ROS (Reactive Oxygen Species)
	Cysteine and methionine metabolism	Amino acid metabolism
	Valine, leucine and isoleucine degradation	These are seen to be elevated in the CF lung – possible phage degradation to increase survival
	Histidine metabolism	Amino acid metabolism
	Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid metabolism
	Pyrimidine metabolism	Metabolizing nucleotide proteins – Cytosine/Thymine/Uracil
	Selenocompound metabolism	Common metabolite found in a range of organisms
	Phenylalanine metabolism	Amino acid metabolism
	Purine metabolism	Nucleotide protein – Adenine/Guanine
> 10 BR	Purine metabolism	Nucleotide protein – Adenine/Guanine
	Pyrimidine metabolism	Nucleotide protein – Cytosine/Thymine/Uracil
	DNA replication	Replication of DNA
	Base excision repair	Involved in Nucleotide Excision Repair
	Nucleotide excision repair	Removal of large damaged amino acids before mutations form
	Homologous recombination	Repairs DSB (Double Strand Breaks)
	beta-Alanine metabolism	Amino acid metabolism
	Pantothenate and CoA biosynthesis	Pantothenate is needed in animals to combine with CoA

		CoA is involved in fatty acid metabolism and the TCA cycle
	Drug metabolism - other enzymes	Drug metabolism
ENERGY METABOLISM	**KEGG Identification**	**Putative function**
Paediatric CF	Oxidative phosphorylation	Release of energy from the mitochondria
	Porphyrin and chlorophyll metabolism	Porphyrin (Heme) and Chlorophyll are energy supplies
< 10 BR	Oxidative phosphorylation	Release of energy from the mitochondria
	Porphyrin and chlorophyll metabolism	Porphyrin (Heme) and Chlorophyll are energy supplies
	Cytochrome bc1 complex respiratory unit	Component in the respiratory chain complex – contains two heme groups and works in electron donating
	Pyruvate metabolism	Glycolysis end product
	Carbon fixation in photosynthetic organisms	Transfer of inorganic Carbon to organic Carbon
	Carbon metabolism	Metabolism of Carbon
	Glyoxylate and dicarboxylate metabolism	Reactions involving glyoxylate and dicarboxylate
	Methane metabolism	Methane metabolism
	Carbon metabolism	Methane metabolism
Adult CF	Oxidative phosphorylation	Release of energy from the mitochondria
> 10 BR	Galactose metabolism	Energy – sugar
	Other glycan degradation	Glycan degradation
	Sphingolipid	Involved in inflammation in

	metabolism	chronic lung diseases
	Starch and sucrose metabolism	Sugar metabolism
	F-type ATPase	Energy generation – ATP
	Oxidative phosphorylation	Release of energy from the mitochondria
	Photosynthesis	Energy supply generated by the mitochondria
XENOBIOTIC DEGRADATION	**KEGG Identification**	**Putative function**
Paediatric CF	Caprolactam degradation	Natural product that is the basis of some drugs
	Dioxin degradation	Heterocyclic 6 membered ring where two Carbons are replaced by oxygen
	Degradation of aromatic compounds	Degradation of amino acids
	Benzene degradation	Benzene ring degradation
	Chlorocyclohexane and chlorobenzene degradation	Chlorobenzene – common chemical, initially DTT (pesticide)
	Fluorobenzoate degradation	Utilize alternative Carbon sources – longevity
	Toluene degradation	Benzene ring derivative – drug related
	Degradation of aromatic compounds	Degradation of aromatic compounds
	Polycyclic aromatic hydrocarbon degradation	Degradation of polycyclic hydrocarbons
< 10 BR	Benzoate degradation	Utilize alternative Carbon sources – longevity
	Dioxin degradation	Heterocyclic 6 membered ring where two Carbons are replaced by oxygen
	Toluene degradation	Benzene ring derivative - drug related
	Degradation of aromatic compounds	Degradation of aromatic compounds

Adult CF	Toluene degradation	Benzene ring derivative - drug related
	Degradation of aromatic compounds	Degradation of aromatic compounds
	Polycyclic aromatic hydrocarbon degradation	Degradation of polycyclic hydrocarbons
	Benzoate degradation	Utilize alternative Carbon sources – longevity
	Chlorocyclohexane and chlorobenzene degradation	Chlorobenzene – common chemical, initially DTT (pesticide)
	Bisphenol degradation	Common chemical exposed to in daily life
	Naphthalene degradation	Double benzene ring product – used in mothballs
	Aminobenzoate degradation	Aminobenzoate has been trialled as a drug previously
	Limonene and pinene degradation	Bronchitis drug – phage can breakdown
	Caprolactam degradation	Natural product that is the basis of some drugs
	Drug metabolism - other enzymes	Drug metabolism
> 10 BR	Chlorocyclohexane and chlorobenzene degradation	Benzene ring degradation
	Chloroalkane and chloroalkene degradation	Chlorobenzene – common chemical, initially DTT (pesticide)
	Atrazine degradation	Degrading chloro derivative alkene's and alkane's
	Caprolactam degradation	Herbicide – benzene ring product
	Toluene degradation	Benzene ring derivative - drug related
	Degradation of aromatic compounds	Degradation of aromatic compounds

MECHANISM OF COFACTORS AND VITAMINS	KEGG Identification	Putative function
Paediatric CF	N/A	
< 10 BR	N/A	
Adult CF	Ubiquinone and other terpenoid-quinone biosynthesis	Involvement in QS (Quorum Sensing)
	Thiamine metabolism	B vitamin – Benzene ring product
	Sulphur relay system	Generation of sulphur – ubiquinated products aid its movement
	Riboflavin metabolism	Vitamin B2, important cofactor in FAD which are essential in pathways such as - fatty acid metabolism, citrate cycle and electron transport chain
	Porphyrin and chlorophyll metabolism	Porphyrin (Heme) and Chlorophyll are energy supplies
> 10 BR	Thiamine metabolism	B vitamin – Benzene ring product
	Sulphur relay system	Generation of sulphur – ubiquinated products aid its movement
METABOLISM OF OTHER AMINO ACIDS	**KEGG Identification**	**Putative function**
Paediatric CF	Arginine and proline metabolism	Amino acid metabolism
	Glutathione metabolism	Antioxidant that protects cells from the damage caused by ROS (Reactive Oxygen Species)
	Lysine biosynthesis	DNA degradation enzyme
	Lysine degradation	Lysine degrades DNA
	Biosynthesis of amino	Biosynthesis of amino acids

	acids	
	Valine, leucine and isoleucine degradation	These are previously seen to be elevated in the CF lung
	Glycine, serine and threonine metabolism	Amino acid metabolism
	Cysteine and methionine metabolism	Amino acid metabolism
	2-Oxocarboxylic acid metabolism	The most elementary set of metabolites -pyruvate (2-oxopropanoate), 2-oxobutanoate, oxaloacetate (2-oxosuccinate) and 2-oxoglutarate
	Pantothenate and CoA biosynthesis	Pantothenate is needed in animals to combine with CoA CoA is involved in fatty acid metabolism and the TCA cycle
	Valine, leucine and isoleucine biosynthesis	Amino acid metabolism – seen in elevated levels in CF lungs
	Alanine, aspartate and glutamate metabolism	Amino acid metabolism
	Two-component system	Response to the environment, allows adaptation to changing environments
	Glutamatergic synapse	Post synaptic synapse – NMDA type
	GABAergic synapse	Neurotransmitter in CNS (Central Nervous System)
< 10 BR	N/A	
Adult CF	Lysine biosynthesis	DNA degradation enzyme
	Peptidoglycan biosynthesis	Sugar metabolism in the cytosol – various enzymes needed

	Histidine metabolism	Amino acid metabolism
	Phenylalanine metabolism	Amino acid metabolism
	Arginine and proline metabolism	Amino acid metabolism
	Taurine and hypotaurine metabolism	Taurine is found in a large proportion of animal tissues, hypotaurine is an intermediate in this process
	Cyanoamino acid metabolism	Amino acid metabolism – addition of a nitrile group to the front of the amino acid
	Glutathione metabolism	Antioxidant that protects cells from the damage caused by ROS (Reactive Oxygen Species)
	Aminoacyl-tRNA biosynthesis	Combines with amino acid whilst they are being synthesized
	Alanine, aspartate and glutamate metabolism	Amino acid metabolism
	Biosynthesis of amino acids	Biosynthesis of amino acids
	Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid metabolism
	Cysteine and methionine metabolism	Amino acid metabolism
> 10 BR	Lysine biosynthesis	DNA degradation enzyme
	2-Oxocarboxylic acid metabolism	The most elementary set of metabolites – pyruvate, oxaloacetate and 2 – oxoglutarate
	Biosynthesis of amino acids	Biosynthesis of amino acids
	Valine, leucine and isoleucine biosynthesis	These are seen to be elevated in the CF lung – possible phage degradation to increase survival

	Pyruvate metabolism	End product of glycolysis
	Arginine and proline metabolism	Amino acid metabolism
	Tryptophan metabolism	Amino acid metabolism
	Glutathione metabolism	Antioxidant that protects cells from the damage caused by ROS (Reactive Oxygen Species)
BIOSYNTHESIS OF OTHER SECONDARY METABOLITES	**KEGG Identification**	**Putative function**
Paediatric CF	N/A	
< 10 BR	Ubiquinone and other terpenoid-quinone biosynthesis	Involvement in QS (Quorum Sensing)
	Porphyrin and chlorophyll metabolism	Porphyrin (Heme) and Chlorophyll are energy supplies
	Drug metabolism - other enzymes	Drug metabolism
	Lysine degradation	Lysine degrades DNA – possible survival tactic
	Biotin metabolism	B vitamin, involved in Gluconeogenesis
Adult CF	N/A	
> 10 BR	Heme biosynthesis	Energy supply
	Porphyrin and chlorophyll metabolism	Porphyrin (Heme) and Chlorophyll are energy supplies
	Ubiquinone biosynthesis	Coenzyme Q10, benzene ring product

ABMAN, S. H., OGLE, J. W., BUTLER-SIMON, N., RUMACK, C. M. & ACCURSO, F. J. 1988. Role of respiratory syncytial virus in early hospitalizations for respiratory distress of young infants with cystic fibrosis. *The Journal of Pediatrics,* 113**,** 826-830.

ACKERMANN, H.-W. 1996. Frequency of morphological phage descriptions in 1995. *Archives of virology,* 141**,** 209-218.

ADAM, B., BAILLIE, G. S. & DOUGLAS, L. J. 2002. Mixed species biofilms of Candida albicans and Staphylococcus epidermidis. *Journal of Medical Microbiology,* 51**,** 344-349.

AHIWALE, S., PRAKASH, D., GAJBHIYE, M., JAGDALE, S., PATIL, N. & KAPADNIS, B. 2012. BVPaP-3, a T7-like lytic phage of pseudomonas aeruginosa: its isolation and characterisation. *Current microbiology,* 64**,** 305-311.

AIRES, J. R., KÖHLER, T., NIKAIDO, H. & PLÉSIAT, P. 1999. Involvement of an active efflux system in the natural resistance of Pseudomonas aeruginosa to aminoglycosides. *Antimicrobial Agents and Chemotherapy,* 43**,** 2624-2628.

AIZENMAN, E., ENGELBERG-KULKA, H. & GLASER, G. 1996. An Escherichia coli chromosomal "addiction module" regulated by guanosine [corrected] 3',5'-bispyrophosphate: a model for programmed bacterial cell death. *Proceedings of the National Academy of Sciences,* 93**,** 6059-6063.

AL-BAKRI, A. G. & AFIFI, F. U. 2007. Evaluation of antimicrobial activity of selected plant extracts by rapid XTT colorimetry and bacterial enumeration. *Journal of Microbiological Methods,* 68**,** 19-25.

ALICE S, P. & NEU, H. C. 1980. Use of piperacillin, a semisynthetic penicillin, in the therapy of acute exacerbations of pulmonary disease in patients with cystic fibrosis. *The Journal of Pediatrics,* 97**,** 148-151.

ALM, R. A., LING, L. S., MOIR, D. T., KING, B. L., BROWN, E. D., DOIG, P. C., et al. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. *Nature,* 397**,** 176-80.

ALMEIDA, M. B. D., ZERBINATI, R. M., TATENO, A. F., OLIVEIRA, C. M., ROMÃO, R. M., RODRIGUES, J. C., et al. 2010. Rhinovirus C and respiratory exacerbations in children with cystic fibrosis. *Emerging infectious diseases,* 16**,** 996-999.

ALTOPARLAK, U., EROL, S., AKCAY, M. N., CELEBI, F. & KADANALI, A. 2004. The time-related changes of antimicrobial resistance patterns and predominant bacterial profiles of burn wounds and body flora of burned patients. *Burns,* 30**,** 660-4.

ANDERSON, C. W. & EIGNER, J. 1971. Breakdown and exclusion of superinfecting T-even bacteriophage in Escherichia coli. *Journal of virology,* 8**,** 869-886.

ANDERSON, D. 1996. Antioxidant defences against reactive oxygen species causing genetic and other damage. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 350**,** 103-108.

ANDREWS, J. M. 2001. The development of the BSAC standardized method of disc diffusion testing. *Journal of Antimicrobial Chemotherapy,* 48**,** 29-42.

ANGLY, F. E., FELTS, B., BREITBART, M., SALAMON, P., EDWARDS, R. A., CARLSON, C., et al. 2006. The marine viromes of four oceanic regions. *PLoS biol,* 4**,** e368.

ANGRILL, J., AGUSTI, C., DE CELIS, R., FILELLA, X., RANO, A., ELENA, M., et al. 2001. Bronchial inflammation and colonization in patients with clinically stable bronchiectasis. *American journal of respiratory and critical care medicine,* 164**,** 1628-1632.

ANGRILL, J., AGUSTI, C., DE CELIS, R., RANO, A., GONZALEZ, J., SOLE, T., et al. 2002. Bacterial colonisation in patients with bronchiectasis: microbiological pattern and risk factors. *Thorax,* 57**,** 15-19.

ARBER, W. 1979. Promotion and limitation of genetic exchange. *Cellular and Molecular Life Sciences,* 35**,** 287-293.

ARMSTRONG, D., GRIMWOOD, K., CARLIN, J. B., CARZINO, R., HULL, J., OLINSKY, A., et al. 1998. Severe viral respiratory infections in infants with cystic fibrosis. *Pediatric pulmonology,* 26**,** 371-379.

ARMSTRONG, D. S., NIXON, G. M., CARZINO, R., BIGHAM, A., CARLIN, J. B., ROBINS-BROWNE, R. M., et al. 2002. Detection of a widespread clone of Pseudomonas aeruginosa in a pediatric cystic fibrosis clinic. *American journal of respiratory and critical care medicine,* 166**,** 983-987.

ARPINO, P., BALDWIN, M. & MCLAFFERTY, F. 1974. Liquid chromatography-mass spectrometry. II—continuous monitoring. *Biological Mass Spectrometry,* 1**,** 80-82.

ARPINO, P. J., GUIOCHON, G., KRIEN, P. & DEVANT, G. 1979. Optimization of the instrumental parameters of a combined liquid chromatograph—mass spectrometer, coupled by an interface for direct liquid introduction: I. Performance of the vacuum equipment. *Journal of Chromatography A,* 185**,** 529-547.

ASHELFORD, K. E., DAY, M. J. & FRY, J. C. 2003. Elevated Abundance of Bacteriophage Infecting Bacteria in Soil. *Applied and Environmental Microbiology,* 69**,** 285-289.

ASHISH, A., PATERSON, S., MOWAT, E., FOTHERGILL, J. L., WALSHAW, M. J. & WINSTANLEY, C. 2013. Extensive diversification is a common feature of Pseudomonas aeruginosa populations during respiratory infections in cystic fibrosis. *Journal of Cystic Fibrosis,* 12**,** 790-793.

ASHISH, A., SHAW, M., WINSTANLEY, C., LEDSON, M. J. & WALSHAW, M. J. 2012. Increasing resistance of the Liverpool Epidemic Strain (LES) of Pseudomonas aeruginosa (Psa) to antibiotics in cystic fibrosis (CF)—A cause for concern? *Journal of Cystic Fibrosis,* 11**,** 173-179.

AVADHANULA, V., RODRIGUEZ, C. A., DEVINCENZO, J. P., WANG, Y., WEBBY, R. J., ULETT, G. C., et al. 2006. Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species-and cell type-dependent manner. *Journal of virology,* 80**,** 1629-1636.

BALTCH, A., SMITH, R., FRANKE, M., RITZ, W., MICHELSEN, P., BOPP, L., et al. 1994. Pseudomonas aeruginosa cytotoxin as a pathogenicity factor in a systemic infection of leukopenic mice. *Toxicon,* 32**,** 27-34.

BANDYOPADHYAY, P. K., STUDIER, F. W., HAMILTON, D. L. & YUAN, R. 1985. Inhibition of the type I restriction-modification enzymes EcoB and EcoK by the gene 0.3 protein of bacteriophage T7. *J Mol Biol,* 182**,** 567-78.

BARER, M. R. & HARWOOD, C. R. 1999. Bacterial viability and culturability. *Adv Microb Physiol,* 41**,** 93-137.

BARKER, A. F. 2002. Bronchiectasis. *New England Journal of Medicine,* 346**,** 1383-1393.

BARR, J. J., YOULE, M. & ROHWER, F. 2013. Innate and acquired bacteriophage-mediated immunity. *Bacteriophage,* 3.

BARRANGOU, R., FREMAUX, C., DEVEAU, H., RICHARDS, M., BOYAVAL, P., MOINEAU, S., et al. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science,* 315**,** 1709-1712.

BAYER, M. E. 1968. Adsorption of Bacteriophages to Adhesions Between Wall and Membrane of Escherichia coli. *Journal of Virology,* 2**,** 346-356.

BEALE, D. J., BARRATT, R., MARLOW, D. R., DUNN, M. S., PALOMBO, E. A., MORRISON, P. D., et al. 2013. Application of metabolomics to understanding biofilms in water distribution systems: a pilot study. *Biofouling,* 29**,** 283-294.

BEDWELL, J., KAIRO, S. & CORBEL, M. 2001. Evaluation of a tetrazolium salt test to determine absence of live mycobacteria in tuberculin purified protein derivative. *Biologicals,* 29**,** 3-6.

BEHNKE, D. & MALKE, H. 1978. Bacteriophage interference in Streptococcus pyogenes I. Characterization of prophage-host systems interfering with the virulent phage A25. *Virology,* 85**,** 118-128.

BELDA-FERRE, P., ALCARAZ, L. D., CABRERA-RUBIO, R., ROMERO, H., SIMON-SORO, A., PIGNATELLI, M., et al. 2012. The oral metagenome in health and disease. *ISME J,* 6**,** 46-56.

BELKUM, A., RENDERS, N. H., SMITH, S., OVERBEEK, S. E. & VERBRUGH, H. A. 2000. Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients1. *FEMS Immunology & Medical Microbiology,* 27**,** 51-57.

BELLIDO, F., MARTIN, N. L., SIEHNEL, R. J. & HANCOCK, R. 1992. Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in Pseudomonas aeruginosa outer membrane permeability. *Journal of bacteriology,* 174**,** 5196-5203.

BENSING, B. A., SIBOO, I. R. & SULLAM, P. M. 2001. Proteins PblA and PblB of Streptococcus mitis, which promote binding to human platelets, are encoded within a lysogenic bacteriophage. *Infection and immunity,* 69**,** 6186-92.

BENTLEY, D. R., BALASUBRAMANIAN, S., SWERDLOW, H. P., SMITH, G. P., MILTON, J., BROWN, C. G., et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. *Nature,* 456**,** 53-9.

BERGER, H. A., ANDERSEN, M. P., GREGORY, R. J., THOMPSON, S., HOWARD, P. W., MAURER, R. A., et al. 1991. Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. *Journal of Clinical Investigation,* 88**,** 1422-1431.

BERGH, Ø., BØRSHEIM, K. Y., BRATBAK, G. & HELDAL, M. 1989. High abundance of viruses found in aquatic environments. *Nature,* 340**,** 467-468.

BERGSTROM, C. T., LO, M. & LIPSITCH, M. 2004. Ecological theory suggests that antimicrobial cycling will not reduce antimicrobial resistance in hospitals. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 13285-13290.

BERTANI, G. 1951. STUDIES ON LYSOGENESIS I.: The Mode of Phage Liberation by Lysogenic Escherichia coli1. *Journal of bacteriology,* 62**,** 293.

BERTANI, G. Lysogenic versus lytic cycle of phage multiplication. Cold Spring Harbor symposia on quantitative biology, 1953. Cold Spring Harbor Laboratory Press, 65-70.

BERTANI, G. 1954. STUDIES ON LYSOGENESIS III.: Superinfection of Lysogenic Shigella dysenteriae with Temperate Mutants of the Carried Phage1. *Journal of bacteriology,* 67**,** 696.

BERTANI, G. The role of phage in bacterial genetics. Brookhaven symposia in biology, 1956. 50-57.

BERTANI, G. 2004. Lysogeny at Mid-Twentieth Century: P1, P2, and Other Experimental Systems. *Journal of Bacteriology,* 186**,** 595-600.

BERTANI, G. & NICE, S. 1954. STUDIES ON LYSOGENESIS II. P1: The Effect of Temperature on the Lysogenization of Shigella Dysenteriae with Phage1. *Journal of bacteriology,* 67**,** 202.

BHAYA, D., DAVISON, M. & BARRANGOU, R. 2011. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annual review of genetics,* 45**,** 273-297.

BITTAR, F., RICHET, H., DUBUS, J.-C., REYNAUD-GAUBERT, M., STREMLER, N., SARLES, J., et al. 2008. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *Journal of Cystic Fibrosis,* 7**,** 4.

BLACKWOOD, L., STONE, R., IGLEWSKI, B. & PENNINGTON, J. 1983. Evaluation of Pseudomonas aeruginosa exotoxin A and elastase as virulence factors in acute lung infection. *Infection and immunity,* 39**,** 198-201.

BLATTNER, F. R., PLUNKETT, G., 3RD, BLOCH, C. A., PERNA, N. T., BURLAND, V., RILEY, M., et al. 1997. The complete genome sequence of Escherichia coli K-12. *Science,* 277**,** 1453-62.

BLOWER, T. R., SHORT, F. L., RAO, F., MIZUGUCHI, K., PEI, X. Y., FINERAN, P. C., et al. 2012. Identification and classification of bacterial Type III toxin-antitoxin systems encoded in chromosomal and plasmid genomes. *Nucleic acids research,* 40**,** 6158-73.

BLUM, G., OTT, M., LISCHEWSKI, A., RITTER, A., IMRICH, H., TSCHÄPE, H., et al. 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an Escherichia coli wild-type pathogen. *Infection and Immunity,* 62**,** 606-614.

BLUMENTHAL, R. M. & CHENG, X. 2002. Restriction-modification systems. *Modern microbial genetics***,** 177-225.

BODEY, G. P., BOLIVAR, R., FAINSTEIN, V. & JADEJA, L. 1983. Infections caused by Pseudomonas aeruginosa. *Review of Infectious Diseases,* 5**,** 279-313.

BOKAREWA, M. I., JIN, T. & TARKOWSKI, A. 2006. Staphylococcus aureus: Staphylokinase. *The international journal of biochemistry & cell biology,* 38**,** 504-9.

BONDY-DENOMY, J., PAWLUK, A., MAXWELL, K. L. & DAVIDSON, A. R. 2013. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature,* 493**,** 429-432.

BOSSO, J. A., SAXON, B., HERBST, J. & MATSEN, J. 1984. Azlocillin pharmacokinetics in patients with cystic fibrosis. *Antimicrobial agents and chemotherapy,* 25**,** 630-632.

BOUCHARD, J. D., DION, E., BISSONNETTE, F. & MOINEAU, S. 2002. Characterization of the two-component abortive phage infection mechanism AbiT from Lactococcus lactis. *Journal of bacteriology,* 184**,** 6325-6332.

BOYD, J. 1951. 'Excessive Dose'Phenomenon in Virus Infections.

BOYTON, R. J. 2012. Bronchiectasis. *Medicine,* 40**,** 267-272.

BRADLEY, D. 1966. The structure and infective process of a Pseudomonas aeruginosa bacteriophage containing ribonucleic acid. *Microbiology,* 45**,** 83-96.

BRADLEY, D. & PITT, T. 1974. Pilus-dependence of four Pseudomonas aeruginosa bacteriophages with non-contractile tails. *Journal of General Virology,* 24**,** 1-15.

BRADLEY, D. E. 1972a. Evidence for the retraction of Pseudomonas aeruginosa RNA phage pili. *Biochemical and biophysical research communications,* 47**,** 142-149.

BRADLEY, D. E. 1972b. Shortening of Pseudomonas aeruginosa pili after RNA-phage adsorption. *Microbiology,* 72**,** 303-319.

BRADLEY, D. E. 1972c. A study of pili on Pseudomonas aeruginosa. *Genet. Res,* 19**,** 39-51.

BRADLEY, D. E. 1973a. The adsorption of the Pseudomonas aeruginosa filamentous bacteriophage Pf to its host. *Canadian journal of microbiology,* 19**,** 623-631.

BRADLEY, D. E. 1973b. Basic characterization of a Pseudomonas aeruginosa pilus-dependent bacteriophage with a long noncontractile tail. *Journal of virology,* 12**,** 1139-1148.

BRADLEY, D. E. 1973c. A pilus-dependent Pseudomonas aeruginosa bacteriophage with a long noncontractile tail. *Virology,* 51**,** 489-492.

BRADLEY, D. E. 1974. The adsorption of Pseudomonas aeruginosa pilus-dependent bacteriophages to a host mutant with nonretractile pili. *Virology,* 58**,** 149-163.

BRADLEY, D. E. 1980. A function of Pseudomonas aeruginosa PAO polar pili: twitching motility. *Canadian journal of microbiology,* 26**,** 146-154.

BRATBAK, G., HELDAL, M., THINGSTAD, T. F. & TUOMI, P. 1996. Dynamics of virus abundance in coastal seawater. *FEMS Microbiology Ecology,* 19**,** 263-269.

BREITBART, M., HAYNES, M., KELLEY, S., ANGLY, F., EDWARDS, R. A., FELTS, B., et al. 2008. Viral diversity and dynamics in an infant gut. *Research in microbiology,* 159**,** 367-373.

BREITBART, M., HEWSON, I., FELTS, B., MAHAFFY, J. M., NULTON, J., SALAMON, P., et al. 2003. Metagenomic analyses of an uncultured viral community from human feces. *Journal of bacteriology,* 185**,** 6220-6223.

BREITBART, M., SALAMON, P., ANDRESEN, B., MAHAFFY, J. M., SEGALL, A. M., MEAD, D., et al. 2002. Genomic analysis of uncultured marine viral communities. *Proceedings of the National Academy of Sciences,* 99**,** 14250-14255.

BRINT, J. M. & OHMAN, D. E. 1995. Synthesis of multiple exoproducts in Pseudomonas aeruginosa is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *Journal of Bacteriology,* 177**,** 7155-7163.

BROCKHURST, M. A., BUCKLING, A. & RAINEY, P. B. 2005. The effect of a bacteriophage on diversification of the opportunistic bacterial pathogen, Pseudomonas aeruginosa. *Proceedings of the Royal Society of London B: Biological Sciences,* 272**,** 1385-1391.

BROUNS, S. J., JORE, M. M., LUNDGREN, M., WESTRA, E. R., SLIJKHUIS, R. J., SNIJDERS, A. P., et al. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science,* 321**,** 960-964.

BROWN, C. T., HOWE, A., ZHANG, Q., PYRKOSZ, A. B. & BROM, T. H. 2012. A reference-free algorithm for computational normalization of shotgun sequencing data. *arXiv preprint arXiv:1203.4802*.

BROWN, N. F., WICKHAM, M. E., COOMBES, B. K. & FINLAY, B. B. 2006. Crossing the line: selection and evolution of virulence traits. *PLoS Pathog,* 2**,** e42.

BRUMFITT, W. & HAMILTON-MILLER, J. M. 1998. Efficacy and safety profile of long-term nitrofurantoin in urinary infections: 18 years' experience. *J Antimicrob Chemother,* 42**,** 363-71.

BRÜSSOW, H., CANCHAYA, C. & HARDT, W.-D. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews,* 68**,** 560-602.

BUCKLING, A. & RAINEY, P. B. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. *Proceedings of the Royal Society B-Biological Sciences,* 269**,** 931-936.

BUELL, C. R., JOARDAR, V., LINDEBERG, M., SELENGUT, J., PAULSEN, I. T., GWINN, M. L., et al. 2003. The complete genome sequence of the Arabidopsis and tomato pathogen Pseudomonas syringae pv. tomato DC3000. *Proceedings of the National Academy of Sciences,* 100**,** 10181-10186.

BULL, J. 1994. Perspective: virulence. *Evolution***,** 1423-1437.

BURKE, V., ROBINSON, J. O., RICHARDSON, C. J. & BUNDELL, C. S. 1991. Longitudinal studies of virulence factors of Pseudomonas aeruginosa in cystic fibrosis. *Pathology,* 23**,** 145-148.

BURNS, N., JAMES, C. E. & HARRISON, E. 2015. Polylysogeny magnifies competitiveness of a bacterial pathogen in vivo. *Evolutionary applications,* 8**,** 346-351.

BUSTAMANTE-CALVILLO, M. E., VELAZQUEZ, F. R., CABRERA-MUNOZ, L., TORRES, J., GOMEZ-DELGADO, A., MORENO, J. A., et al. 2001. Molecular detection of respiratory syncytial virus in postmortem lung tissue samples from Mexican children deceased with pneumonia. *Pediatr Infect Dis J,* 20**,** 495-501.

BYRNE, M. & KROPINSKI, A. M. 2005. The genome of the< i> Pseudomonas aeruginosa</i> generalized transducing bacteriophage F116. *Gene,* 346**,** 187-194.

CADY, K. C., BONDY-DENOMY, J., HEUSSLER, G. E., DAVIDSON, A. R. & O'TOOLE, G. A. 2012. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. *Journal of bacteriology,* 194**,** 5728-5738.

CAI, L., YE, L., TONG, A. H. Y., LOK, S. & ZHANG, T. 2013. Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. *PLoS One,* 8**,** e53649.

CALLAGHAN, M. & MCCLEAN, S. 2012. Bacterial host interactions in cystic fibrosis. *Current opinion in microbiology,* 15**,** 71-77.

CAMPOCCIA, D., MONTANARO, L., BALDASSARRI, L., AN, Y. H. & ARCIOLA, C. R. 2005. Antibiotic resistance in Staphylococcus aureus and Staphylococcus epidermidis clinical isolates from implant orthopedic infections. *Int J Artif Organs,* 28**,** 1186-91.

CANT, N., POLLOCK, N. & FORD, R. C. 2014. CFTR structure and cystic fibrosis. *The International Journal of Biochemistry & Cell Biology,* 52**,** 15-25.

CASTRIC, P. A. & DEAL, C. D. 1994. Differentiation of Pseudomonas aeruginosa pili based on sequence and B-cell epitope analyses. *Infection and immunity,* 62**,** 371-376.

CAZARES, A., MENDOZA-HERNÁNDEZ, G. & GUARNEROS, G. 2014. Core and accessory genome architecture in a group of Pseudomonas aeruginosa Mu-like phages. *BMC Genomics,* 15**,** 1-13.

CERCA, N., MARTINS, S., CERCA, F., JEFFERSON, K. K., PIER, G. B., OLIVEIRA, R., et al. 2005. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. *Journal of Antimicrobial Chemotherapy,* 56**,** 331-336.

CERDEÑO-TÁRRAGA, A. M., EFSTRATIOU, A., DOVER, L. G., HOLDEN, M. T. G., PALLEN, M., BENTLEY, S. D., et al. 2003. The complete genome sequence and analysis of Corynebacterium diphtheriae NCTC13129. *Nucleic acids research,* 31**,** 6516-6523.

CEYSSENS, P.-J. & LAVIGNE, R. 2010. Bacteriophages of Pseudomonas. *Future Microbiology,* 5**,** 1041-1055.

CEYSSENS, P.-J., LAVIGNE, R., MATTHEUS, W., CHIBEU, A., HERTVELDT, K., MAST, J., et al. 2006. Genomic analysis of Pseudomonas aeruginosa phages LKD16 and LKA1: Establishment of the ϕKMV subgroup within the T7 supergroup. *Journal of Bacteriology,* 188**,** 6924-6931.

CHAKRAVORTY, S., HELB, D., BURDAY, M., CONNELL, N. & ALLAND, D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of microbiological methods,* 69**,** 330-339.

CHAMBERS, D., SCOTT, F., BANGUR, R., DAVIES, R., LIM, A., WALTERS, S., et al. 2005. Factors associated with infection by Pseudomonas aeruginosa in adult cystic fibrosis. *European Respiratory Journal,* 26**,** 651-656.

CHANCE, B., SIES, H. & BOVERIS, A. 1979. Hydroperoxide metabolism in mammalian organs. *Physiol Rev,* 59**,** 527-605.

CHEN, F. & LU, J. 2002. Genomic sequence and evolution of marine cyanophage P60: a new insight on lytic and lysogenic phages. *Applied and Environmental Microbiology,* 68**,** 2589-2594.

CHEN, J., CARPENA, N., QUILES-PUCHALT, N., RAM, G., NOVICK, R. P. & PENADES, J. R. 2015. Intra-and inter-generic transfer of pathogenicity island-encoded virulence genes by cos phages. *ISME Journal,* 9**,** 1260-1263.

CHEN, J. & NOVICK, R. P. 2009. Phage-mediated intergeneric transfer of toxin genes. *science,* 323**,** 139-141.

CHENG, K., SMYTH, R. L., GOVAN, J. R., DOHERTY, C., WINSTANLEY, C., DENNING, N., et al. 1996. Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic. *Lancet,* 348**,** 639-42.

CHERNY, I. & GAZIT, E. 2004. The YefM antitoxin defines a family of natively unfolded proteins: Implications as a novel antibacterial target. *Journal of Biological Chemistry,* 279**,** 8252-8261.

CHIBANI-CHENNOUFI, S., BRUTTIN, A., DILLMANN, M.-L. & BRÜSSOW, H. 2004. Phage-host interaction: an ecological perspective. *Journal of bacteriology,* 186**,** 3677-3686.

CHOKKATHUKALAM, A., KIM, D.-H., BARRETT, M. P., BREITLING, R. & CREEK, D. J. 2014. Stable isotope-labeling studies in metabolomics: new insights into structure and dynamics of metabolic networks. *Bioanalysis,* 6**,** 511-524.

CHOPIN, M.-C., CHOPIN, A. & BIDNENKO, E. 2005. Phage abortive infection in lactococci: variations on a theme. *Current Opinion in Microbiology,* 8**,** 473-479.

CHOWDHURY, R., BISWAS, S. K. & DAS, J. 1989. Abortive replication of choleraphage Φ 149 in Vibrio cholerae biotype El Tor. *Journal of virology,* 63**,** 392-397.

CHRISTENSEN, S. K., MAENHAUT-MICHEL, G., MINE, N., GOTTESMAN, S., GERDES, K. & VAN MELDEREN, L. 2004. Overproduction of the Lon protease triggers inhibition of translation in Escherichia coli: Involvement of the yefM-yoeB toxin-antitoxin system. *Molecular Microbiology,* 51**,** 1705-1717.

CHRISTENSEN, S. K., MIKKELSEN, M., PEDERSEN, K. & GERDES, K. 2001. RelE, a global inhibitor of translation, is activated during nutritional stress. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 14328-14333.

CHRISTENSEN, S. K., PEDERSEN, K., HANSEN, F. G. & GERDES, K. 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *Journal of Molecular Biology,* 332**,** 809-819.

CHURCH, D., ELSAYED, S., REID, O., WINSTON, B. & LINDSAY, R. 2006. Burn wound infections. *Clin Microbiol Rev,* 19**,** 403-34.

CIOFU, O., FUSSING, V., BAGGE, N., KOCH, C. & HØIBY, N. 2001. Characterization of paired mucoid/non-mucoid Pseudomonas aeruginosa isolates from Danish cystic fibrosis patients: antibiotic resistance, β-lactamase activity and RiboPrinting. *Journal of Antimicrobial Chemotherapy,* 48**,** 391-396.

CLOWES, R. C. 1972. Molecular structure of bacterial plasmids. *Bacteriological Reviews,* 36**,** 361.

COENYE, T., VANDAMME, P., GOVAN, J. R. & LIPUMA, J. J. 2001. Taxonomy and identification of the Burkholderia cepacia complex. *Journal of Clinical Microbiology,* 39**,** 3427-3436.

COETZEE, J. 1987. Phage Ecology. Wiley and Sons Inc. New York.

COHEN, M. & SAHN, S. A. 1999. Bronchiectasis in systemic diseases. *CHEST Journal,* 116**,** 1063-1074.

COLE, P. 1985. Inflammation: a two-edged sword--the model of bronchiectasis. *European journal of respiratory diseases. Supplement,* 147**,** 6-15.

COLEMAN, D. C., SULLIVAN, D. J., RUSSELL, R. J., ARBUTHNOTT, J. P., CAREY, B. F. & POMEROY, H. M. 1989. Staphylococcus aureus bacteriophages mediating the simultaneous lysogenic conversion of beta-lysin, staphylokinase and enterotoxin A: molecular mechanism of triple conversion. *Journal of general microbiology,* 135**,** 1679-97.

COLLINSON, J., NICHOLSON, K. G., CANCIO, E., ASHMAN, J., IRELAND, D. C., HAMMERSLEY, V., et al. 1996. Effects of upper respiratory tract infections in patients with cystic fibrosis. *Thorax,* 51**,** 1115-1122.

COMEAU, A. M. & KRISCH, H. M. 2005. War is peace — dispatches from the bacterial and phage killing fields. *Current Opinion in Microbiology,* 8**,** 488-494.

CORNELIS, P. 2008. *Pseudomonas: genomics and molecular biology*, Horizon Scientific Press.

COSTERTON, J. W. 1995. Overview of microbial biofilms. *J Ind Microbiol,* 15**,** 137-40.

COSTERTON, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. & LAPPIN-SCOTT, H. M. 1995. Microbial biofilms. *Annual Reviews in Microbiology,* 49**,** 711-745.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. 1999. Bacterial biofilms: a common cause of persistent infections. *Science,* 284**,** 1318-1322.

COVEY, T. R., LEE, E. D., BRUINS, A. P. & HENION, J. D. 1986. Liquid chromatography/mass spectrometry. *Analytical Chemistry,* 58**,** 1451A-1461A.

CRAIG, D. W., PEARSON, J. V., SZELINGER, S., SEKAR, A., REDMAN, M., CORNEVEAUX, J. J., et al. 2008. Identification of genetic variants using bar-coded multiplexed sequencing. *Nature methods,* 5**,** 887-893.

CUMBY, N., EDWARDS, A. M., DAVIDSON, A. R. & MAXWELL, K. L. 2012. The Bacteriophage HK97 gp15 Moron Element Encodes a Novel Superinfection Exclusion Protein. *Journal of Bacteriology,* 194**,** 5012-5019.

CUNHA, B. A. 2006. New uses for older antibiotics: nitrofurantoin, amikacin, colistin, polymyxin B, doxycycline, and minocycline revisited. *Med Clin North Am,* 90**,** 1089-107.

CUNHA, B. A., SCHOCH, P. E. & HAGE, J. R. 2011. Nitrofurantoin: Preferred Empiric Therapy for Community-Acquired Lower Urinary Tract Infections. *Mayo Clinic Proceedings,* 86**,** 1243-1244.

CURRAN, B., JONAS, D., GRUNDMANN, H., PITT, T. & DOWSON, C. G. 2004. Development of a multilocus sequence typing scheme for the opportunistic pathogen Pseudomonas aeruginosa. *Journal of Clinical Microbiology,* 42**,** 5644-5649.

D'HERELLE, F. 1917. Sur un microbe invisible antagoniste des bacilles dysentériques. *CR Acad. Sci. Paris,* 165**,** 373-375.

DAJANI, A. S. 1972. The scalded-skin syndrome: relation to phage-group II staphylococci. *Journal of Infectious Diseases,* 125**,** 548-551.

DAMRON, F. H., DAVIS JR, M. R., WITHERS, T. R., ERNST, R. K., GOLDBERG, J. B., YU, G., et al. 2011. Vanadate and triclosan synergistically induce alginate production by Pseudomonas aeruginosa strain PAO1. *Molecular microbiology,* 81**,** 554-570.

DAMRON, F. H. & GOLDBERG, J. B. 2012. Proteolytic regulation of alginate overproduction in Pseudomonas aeruginosa. *Molecular microbiology,* 84**,** 595-607.

DART, J. K. & SEAL, D. V. 1988. Pathogenesis and therapy of Pseudomonas aeruginosa keratitis. *Eye,* 2 Suppl**,** S46-55.

DASGUPTA, T., DE KIEVIT, T. R., MASOUD, H., ALTMAN, E., RICHARDS, J. C., SADOVSKAYA, I., et al. 1994. Characterization of lipopolysaccharide-deficient

mutants of Pseudomonas aeruginosa derived from serotypes O3, O5, and O6. *Infection and immunity,* 62**,** 809-17.

DAVIES, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nature reviews Drug discovery,* 2**,** 114-122.

DAVIES, D. G., PARSEK, M. R., PEARSON, J. P., IGLEWSKI, B. H., COSTERTON, J. & GREENBERG, E. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science,* 280**,** 295-298.

DAVIES, E. V., JAMES, C. E., KUKAVICA-IBRULJ, I., LEVESQUE, R. C., BROCKHURST, M. A. & WINSTANLEY, C. 2016a. Temperate phages enhance pathogen fitness in chronic lung infection. *ISME J*.

DAVIES, E. V., JAMES, C. E., WILLIAMS, D., O'BRIEN, S., FOTHERGILL, J. L., HALDENBY, S., et al. 2016b. Temperate phages both mediate and drive adaptive evolution in pathogen biofilms. *Proceedings of the National Academy of Sciences*.

DAVIS, P. B., DRUMM, M. & KONSTAN, M. W. 1996. Cystic fibrosis. *Am J Respir Crit Care Med,* 154**,** 1229-56.

DAWKINS, R. & KREBS, J. R. 1979. Arms races between and within species. *Proceedings of the Royal Society of London B: Biological Sciences,* 205**,** 489-511.

DAY, W. A., FERNÁNDEZ, R. E. & MAURELLI, A. T. 2001. Pathoadaptive Mutations That Enhance Virulence: Genetic Organization of the cadA Regions ofShigella spp. *Infection and immunity,* 69**,** 7471-7480.

DE GROOTE, M. A., OCHSNER, U. A., SHILOH, M. U., NATHAN, C., MCCORD, J. M., DINAUER, M. C., et al. 1997. Periplasmic superoxide dismutase protects Salmonella from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proceedings of the National Academy of Sciences,* 94**,** 13997-14001.

DE KIEVIT, T. R. & IGLEWSKI, B. H. 2000. Bacterial quorum sensing in pathogenic relationships. *Infection and Immunity,* 68**,** 4839-4849.

DELMONT, T. O., ROBE, P., CECILLON, S., CLARK, I. M., CONSTANCIAS, F., SIMONET, P., et al. 2011. Accessing the Soil Metagenome for Studies of Microbial Diversity. *Applied and Environmental Microbiology,* 77**,** 1315-1324.

DELTCHEVA, E., CHYLINSKI, K., SHARMA, C. M., GONZALES, K., CHAO, Y., PIRZADA, Z. A., et al. 2011. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature,* 471**,** 602-607.

DENAMUR, E., PICARD, B., DECOUX, G., DENIS, J.-B. & ELION, J. 1993. The absence of correlation between allozyme and rrn RFLP analysis indicates a high gene flow rate within human clinical Pseudomonas aeruginosa isolates. *FEMS microbiology letters,* 110**,** 275-280.

DENTON, M., KERR, K., MOONEY, L., KEER, V., RAJGOPAL, A., BROWNLEE, K., et al. 2002. Transmission of colistin-resistant Pseudomonas aeruginosa between patients attending a pediatric cystic fibrosis center. *Pediatric pulmonology,* 34**,** 257-261.

DERETIC, V., SCHURR, M., BOUCHER, J. & MARTIN, D. 1994. Conversion of Pseudomonas aeruginosa to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *Journal of bacteriology,* 176**,** 2773.

DESIERE, F., MCSHAN, W. M., VAN SINDEREN, D., FERRETTI, J. J. & BRÜSSOW, H. 2001. Comparative Genomics Reveals Close Genetic Relationships between Phages from Dairy Bacteria and Pathogenic Streptococci: Evolutionary Implications for Prophage-Host Interactions. *Virology,* 288**,** 325-341.

DESNUES, C., RODRIGUEZ-BRITO, B., RAYHAWK, S., KELLEY, S., TRAN, T., HAYNES, M., et al. 2008. Biodiversity and biogeography of phages in modern stromatolites and thrombolites. *Nature,* 452**,** 340-343.

DEVEAU, H., BARRANGOU, R., GARNEAU, J. E., LABONTE, J., FREMAUX, C., BOYAVAL, P., et al. 2008. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *Journal of bacteriology,* 190**,** 1390-400.

DEVEAU, H., GARNEAU, J. E. & MOINEAU, S. 2010. CRISPR/Cas system and its role in phage-bacteria interactions. *Annual review of microbiology,* 64**,** 475-493.

DEZIEL, E., COMEAU, Y. & VILLEMUR, R. 2001. Initiation of biofilm formation by Pseudomonas aeruginosa 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol,* 183**,** 1195-204.

DHILLON, E. K., DHILLON, T. S., LAI, A. N. & LINN, S. 1980. Host range, immunity and antigenic properties of lambdoid coliphage HK97. *Journal of General Virology,* 50**,** 217-220.

DHILLON, T. S. & DHILLON, E. K. S. 1976. Temperate coliphage HK022. Clear plaque mutants and preliminary vegetative map. *Japanese Journal of Microbiology,* 20**,** 385-396.

DINSDALE, E. A., EDWARDS, R. A., HALL, D., ANGLY, F., BREITBART, M., BRULC, J. M., et al. 2008. Functional metagenomic profiling of nine biomes. *Nature,* 452**,** 629-632.

DOGGETT, R. G. 1969. Incidence of mucoid Pseudomonas aeruginosa from clinical sources. *Applied microbiology,* 18**,** 936-937.

DOGGETT, R. G., HARRISON, G. M., STILLWELL, R. N. & WALLIS, E. S. 1966. An atypical Pseudomonas aeruginosa associated with cystic fibrosis of the pancreas. *The Journal of Pediatrics,* 68**,** 215-221.

DOGGETT, R. G., HARRISON, G. M. & WALLIS, E. S. 1964. Comparison of some properties of Pseudomonas aeruginosa isolated from infections in persons with and without cystic fibrosis. *Journal of bacteriology,* 87**,** 427-431.

DORMANN, C. F., FRÜND, J., BLÜTHGEN, N. & GRUBER, B. 2009. Indices, graphs and null models: analyzing bipartite ecological networks.

DORMANN, C. F., GRUBER, B. & FRÜND, J. 2008. Introducing the bipartite package: analysing ecological networks. *interaction,* 1**,** 0.2413793.

DRAWZ, S. M., PAPP-WALLACE, K. M. & BONOMO, R. A. 2014. New β-Lactamase Inhibitors: a Therapeutic Renaissance in an MDR World. *Antimicrobial Agents and Chemotherapy,* 58**,** 1835-1846.

DRENKARD, E. & AUSUBEL, F. M. 2002a. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature,* 416**,** 740-3.

DRENKARD, E. & AUSUBEL, F. M. 2002b. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature,* 416**,** 740-743.

DRYDEN, D. T. & TOCK, M. R. 2006. DNA mimicry by proteins. *Biochem Soc Trans,* 34**,** 317-9.

DUBERN, J.-F. & DIGGLE, S. P. 2008. Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species. *Molecular Biosystems,* 4**,** 882-888.

DULBECCO, R. 1952. Mutual exclusion between related phages. *Journal of bacteriology,* 63**,** 209.

DUNN, W. B., ERBAN, A., WEBER, R. J., CREEK, D. J., BROWN, M., BREITLING, R., et al. 2013. Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. *Metabolomics,* 9**,** 44-66.

EDENBOROUGH, F., STONE, H., KELLY, S., ZADIK, P., DOHERTY, C. & GOVAN, J. 2004. Genotyping of Pseudomonas aeruginosa in cystic fibrosis suggests need for segregation. *Journal of Cystic Fibrosis,* 3**,** 37-44.

EDLIN, G., LIN, L. & BITNER, R. 1977. Reproductive fitness of P1, P2, and Mu lysogens of Escherichia coli. *Journal of virology,* 21**,** 560-564.

EDLIN, G., LIN, L. & KUDRNA, R. 1975. λ Lysogens of E. coli reproduce more rapidly than non-lysogens.

EDWARDS, R. A. & ROHWER, F. 2005. Viral metagenomics. *Nature Reviews Microbiology,* 3**,** 504-510.

EKLUND, M. W., POYSKY, F. T. & REED, S. M. 1972. Bacteriophage and the toxigenicity of Clostridium botulinum type D. *Nature: New biology,* 235**,** 16-7.

EKLUND, M. W., POYSKY, F. T., REED, S. M. & SMITH, C. A. 1971. Bacteriophage and the toxigenicity of Clostridium botulinum type C. *Science,* 172**,** 480-2.

ELLIS, D. I., DUNN, W. B., GRIFFIN, J. L., ALLWOOD, J. W. & GOODACRE, R. 2007. Metabolic fingerprinting as a diagnostic tool. *Pharmacogenomics,* 8**,** 1243-66.

ENRIGHT, M. C., ROBINSON, D. A., RANDLE, G., FEIL, E. J., GRUNDMANN, H. & SPRATT, B. G. 2002. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). *Proceedings of the National Academy of Sciences,* 99**,** 7687-7692.

ERAM, S. M., NEJAD, Q. B., KHATAMI, G.-R. & NAFISSI, N. 2004. Detection of Burkholderia cepacia complex in patients with cystic fibrosis. *Tanaffos,* 3**,** 47-52.

ERGON, R. 2005. PLS post-processing by similarity transformation (PLS + ST): a simple alternative to OPLS. *Journal of Chemometrics,* 19**,** 1-4.

ESTAHBANATI, H. K., KASHANI, P. P. & GHANAATPISHEH, F. 2002. Frequency of Pseudomonas aeruginosa serotypes in burn wound infections and their resistance to antibiotics. *Burns,* 28**,** 340-348.

FANG, F. C., DEGROOTE, M. A., FOSTER, J. W., BÄUMLER, A. J., OCHSNER, U., TESTERMAN, T., et al. 1999. Virulent Salmonella typhimurium has two periplasmic Cu, Zn-superoxide dismutases. *Proceedings of the National Academy of Sciences,* 96**,** 7502-7507.

FARRANT, J. L., SANSONE, A., CANVIN, J. R., PALLEN, M. J., LANGFORD, P. R., WALLIS, T. S., et al. 1997. Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. *Molecular Microbiology,* 25**,** 785-796.

FEARY, T. W., FISHER, E. & FISHER, T. N. 1964. Isolation and preliminary characteristics of three bacteriophages associated with a lysogenic strain of Pseudomonas aeruginosa. *Journal of bacteriology,* 87**,** 196-208.

FERRETTI, J. J., MCSHAN, W. M., AJDIC, D., SAVIC, D. J., SAVIC, G., LYON, K., et al. 2001. Complete genome sequence of an M1 strain of Streptococcus pyogenes. *Proceedings of the National Academy of Sciences,* 98**,** 4658-4663.

FERRONI, A., NGUYEN, L., PRON, B., QUESNE, G., BRUSSET, M. C. & BERCHE, P. 1998. Outbreak of nosocomial urinary tract infections due to Pseudomonas aeruginosa in a paediatric surgical unit associated with tap-water contamination. *Journal of Hospital Infection,* 39**,** 301-307.

FIEHN, O. 2002. Metabolomics–the link between genotypes and phenotypes. *Plant molecular biology,* 48**,** 155-171.

FIGUEROA-BOSSI, N. & BOSSI, L. 1999. Inducible prophages contribute to Salmonella virulence in mice. *Molecular Microbiology,* 33**,** 167-176.

FIGUEROA-BOSSI, N., COISSAC, E., NETTER, P. & BOSSI, L. 1997. Unsuspected prophage-like elements in Salmonella typhimurium. *Molecular Microbiology,* 25**,** 161-173.

FIGUEROA-BOSSI, N., UZZAU, S., MALORIOL, D. & BOSSI, L. 2001. Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in Salmonella. *Molecular microbiology,* 39**,** 260-272.

FILKINS, L. M., HAMPTON, T. H., GIFFORD, A. H., GROSS, M. J., HOGAN, D. A., SOGIN, M. L., et al. 2012. Prevalence of Streptococci and Increased Polymicrobial Diversity

Associated with Cystic Fibrosis Patient Stability. *Journal of Bacteriology,* 194**,** 4709-4717.

FINERAN, P. C., BLOWER, T. R., FOULDS, I. J., HUMPHREYS, D. P., LILLEY, K. S. & SALMOND, G. P. 2009. The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 894-9.

FLEISZIG, S. M. J. & EVANS, D. J. 2002. The pathogenesis of bacterial keratitis: studies with Pseudomonas aeruginosa. *Clinical and Experimental Optometry,* 85**,** 271-278.

FOGG, P., RIGDEN, D., SAUNDERS, J., MCCARTHY, A. & ALLISON, H. 2010. Characterization of the relationship between integrase, excisionase and antirepressor activities associated with a superinfecting Shiga toxin encoding bacteriophage. *Nucleic acids research***,** gkq923.

FOGG, P. C., GOSSAGE, S. M., SMITH, D. L., SAUNDERS, J. R., MCCARTHY, A. J. & ALLISON, H. E. 2007. Identification of multiple integration sites for Stx-phage Phi24B in the Escherichia coli genome, description of a novel integrase and evidence for a functional anti-repressor. *Microbiology,* 153**,** 4098-110.

FOGG, P. C. M., COLLOMS, S., ROSSER, S., STARK, M. & SMITH, M. C. M. 2014. New Applications for Phage Integrases. *Journal of Molecular Biology,* 426**,** 2703-2716.

FONSECA, A., EXTREMINA, C., FONSECA, A. & SOUSA, J. 2004. Effect of subinhibitory concentration of piperacillin/tazobactam on Pseudomonas aeruginosa. *Journal of medical microbiology,* 53**,** 903-910.

FORD, M. E., SARKIS, G. J., BELANGER, A. E., HENDRIX, R. W. & HATFULL, G. F. 1998a. Genome structure of mycobacteriophage D29: implications for phage evolution. *Journal of molecular biology,* 279**,** 143-164.

FORD, M. E., STENSTROM, C., HENDRIX, R. W. & HATFULL, G. F. 1998b. Mycobacteriophage TM4: genome structure and gene expression. *Tubercle and Lung Disease,* 79**,** 63-73.

FORDE, A. & FITZGERALD, G. 1999. Bacteriophage defence systems in lactic acid bacteria. *In:* KONINGS, W. N., KUIPERS, O. P. & IN 'T VELD, J. H. J. H. (eds.) *Lactic Acid Bacteria: Genetics, Metabolism and Applications.* Springer Netherlands.

FORTIER, L. C. & SEKULOVIC, O. 2013. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence,* 4.

FOTHERGILL, J. L., MOWAT, E., LEDSON, M. J., WALSHAW, M. J. & WINSTANLEY, C. 2010. Fluctuations in phenotypes and genotypes within populations of Pseudomonas aeruginosa in the cystic fibrosis lung during pulmonary exacerbations. *J Med Microbiol,* 59**,** 472-81.

FOTHERGILL, J. L., MOWAT, E., WALSHAW, M. J., LEDSON, M. J., JAMES, C. E. & WINSTANLEY, C. 2011. Effect of antibiotic treatment on bacteriophage production by a cystic fibrosis epidemic strain of Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy,* 55**,** 426-8.

FOTHERGILL, J. L., WALSHAW, M. J. & WINSTANLEY, C. 2012. Transmissible strains of Pseudomonas aeruginosa in cystic fibrosis lung infections. *European Respiratory Journal,* 40**,** 227-238.

FOWERAKER, J., LAUGHTON, C., BROWN, D. & BILTON, D. 2005. Phenotypic variability of Pseudomonas aeruginosa in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. *Journal of Antimicrobial Chemotherapy,* 55**,** 921-927.

FOWERAKER, J. E., LAUGHTON, C. R., BROWN, D. F. & BILTON, D. 2009. Comparison of methods to test antibiotic combinations against heterogeneous populations of

multiresistant Pseudomonas aeruginosa from patients with acute infective exacerbations in cystic fibrosis. *Antimicrob Agents Chemother,* 53**,** 4809-15.

FRIEDMAN, L. & KOLTER, R. 2004a. Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms. *Molecular microbiology,* 51**,** 675-690.

FRIEDMAN, L. & KOLTER, R. 2004b. Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix. *Journal of bacteriology,* 186**,** 4457-4465.

FRIZZELL, R. A. 1995. Function of the cystic fibrosis transmembrane conductance regulator protein. *American journal of respiratory and critical care medicine,* 151**,** S54.

FRY, B. 1959. Conditions for the infection of Escherichia coli with lambda phage and for the establishment of lysogeny. *Journal of general microbiology,* 21**,** 676-684.

FUHRMAN, J. A. 1999. Marine viruses and their biogeochemical and ecological effects. *Nature,* 399**,** 541-548.

FUJIMOTO, D., SRINIVASAN, P. & BOREK, E. 1965. On the Nature of the Deoxyribonucleic Acid Methylases. Biological Evidence for the Multiple Nature of the Enzymes*. *Biochemistry,* 4**,** 2849-2855.

FULLER, N. J., WILSON, W. H., JOINT, I. R. & MANN, N. H. 1998. Occurrence of a sequence in marine cyanophages similar to that of T4 g20 and its application to PCR-based detection and quantification techniques. *Applied and Environmental Microbiology,* 64**,** 2051-2060.

GABRIELSON, J., HART, M., JARELÖV, A., KÜHN, I., MCKENZIE, D. & MÖLLBY, R. 2002. Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. *Journal of Microbiological Methods,* 50**,** 63-73.

GALÁN, J. E. & CURTISS, R. 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. *Proceedings of the National Academy of Sciences,* 86**,** 6383-6387.

GAMA, J. A., REIS, A. M., DOMINGUES, I., MENDES-SOARES, H., MATOS, A. M. & DIONISIO, F. 2013. Temperate bacterial viruses as double-edged swords in bacterial warfare. *PloS one,* 8**,** e59043.

GAMBELLO, M. J., KAYE, S. & IGLEWSKI, B. H. 1993. LasR of Pseudomonas aeruginosa is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infection and immunity,* 61**,** 1180-1184.

GARBE, J., BUNK, B., ROHDE, M. & SCHOBERT, M. 2011. Sequencing and Characterization of Pseudomonas aeruginosa phage JG004. *BMC Microbiology,* 11**,** 1-12.

GELLATLY, S. L. & HANCOCK, R. E. 2013. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathogens and disease,* 67**,** 159-173.

GEORGE, J., CASTELLAZZI, M. & BUTTIN, G. 1975. Prophage induction and cell division in E. coli. *Molecular and General Genetics MGG,* 140**,** 309-332.

GERM, M., YOSHIHARA, E., YONEYAMA, H. & NAKAE, T. 1999. Interplay between the efflux pump and the outer membrane permeability barrier in fluorescent dye accumulation in Pseudomonas aeruginosa. *Biochemical and biophysical research communications,* 261**,** 452-455.

GIBSON, R. L., BURNS, J. L. & RAMSEY, B. W. 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. *American journal of respiratory and critical care medicine,* 168**,** 918-951.

GILBERT, W. & MAXAM, A. 1973. The Nucleotide Sequence of the lac Operator. *Proceedings of the National Academy of Sciences,* 70**,** 3581-3584.

GILBOA-GARBER, N. 1983. The biological functions of Pseudomonas aeruginosa lectins. *Lectins,* 3**,** 495-502.

GILBOA-GARBER, N. 1986. Lectins of Pseudomonas aeruginosa: properties, biological effects and applications. *Microbial Lectins and Agglutinins: Properties and Biological Activity. John Wiley & Sons, New York***,** 255-269.

GILBOA-GARBER, N. 1997. Multiple aspects of Pseudomonas aeruginosa lectins. *Nova Acta Leopoldina NF,* 75**,** 153-177.

GILBOA-GARBER, N., KATCOFF, D. J. & GARBER, N. C. 2000. Identification and characterization of Pseudomonas aeruginosa PA-IIL lectin gene and protein compared to PA-IL. *FEMS Immunology and Medical Microbiology,* 29**,** 53-57.

GILBOA-GARBER, N., MIZRAHI, L. & GARBER, N. 1972. Purification of the galactose-binding hemagglutinin of Pseudomonas aeruginosa by affinity column chromatography using sepharose. *FEBS Letters,* 28**,** 93-95.

GILL, S. R., FOUTS, D. E., ARCHER, G. L., MONGODIN, E. F., DEBOY, R. T., RAVEL, J., et al. 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant Staphylococcus aureus strain and a biofilm-producing methicillin-resistant Staphylococcus epidermidis strain. *Journal of bacteriology,* 187**,** 2426-2438.

GJERSING, E. L., HERBERG, J. L., HORN, J., SCHALDACH, C. M. & MAXWELL, R. S. 2007. NMR metabolomics of planktonic and biofilm modes of growth in Pseudomonas aeruginosa. *Analytical chemistry,* 79**,** 8037-8045.

GOERKE, C., PANTUCEK, R., HOLTFRETER, S., SCHULTE, B., ZINK, M., GRUMANN, D., et al. 2009. Diversity of prophages in dominant Staphylococcus aureus clonal lineages. *Journal of bacteriology,* 191**,** 3462-3468.

GOLD, M., HAUSMANN, R., MAITRA, U. & HURWITZ, J. 1964. The enzymatic methylation of RNA and DNA, VIII. Effects of bacteriophage infection on the activity of the methylating enzymes. *Proceedings of the National Academy of Sciences,* 52**,** 292-297.

GÓMEZ, P. & BUCKLING, A. 2011. Bacteria-phage antagonistic coevolution in soil. *Science,* 332**,** 106-109.

GOODMAN, B. E. & PERCY, W. H. 2005. CFTR in cystic fibrosis and cholera: from membrane transport to clinical practice. *Advances in physiology education,* 29**,** 75-82.

GOVAN, J., DOHERTY, C., NELSON, J., BROWN, P., GREENING, A., MADDISON, J., et al. 1993. Evidence for transmission of Pseudomonas cepacia by social contact in cystic fibrosis. *The Lancet,* 342**,** 15-19.

GOVAN, J. R. & DERETIC, V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiological reviews,* 60**,** 539-574.

GRAHAM, A. The fate of the infecting phage particle. Annales de l'Institut Pasteur, 1953. 90.

GREEN, M. R. & SAMBROOK, J. 2012. Molecular cloning: a laboratory manual.

GROOT, R., HACK, B. D., WEBER, A., CHAFFIN, D., RAMSEY, B. & SMITH, A. L. 1990. Pharmacokinetics of ticarcillin in patients with cystic fibrosis: a controlled prospective study. *Clinical Pharmacology & Therapeutics,* 47**,** 73-78.

GUIXA-BOIXEREU, N., VAQUÉ, D., GASOL, J. M., SÁNCHEZ-CÁMARA, J. & PEDRÓS-ALIÓ, C. 2002. Viral distribution and activity in Antarctic waters. *Deep Sea Research Part II: Topical Studies in Oceanography,* 49**,** 827-845.

GUO, A. C., JEWISON, T., WILSON, M., LIU, Y., KNOX, C., DJOUMBOU, Y., et al. 2013. ECMDB: the E. coli Metabolome Database. *Nucleic Acids Res,* 41**,** D625-30.

HAHN, H. P. 1997. The type-4 pilus is the major virulence-associated adhesin of Pseudomonas aeruginosa – a review12. *Gene,* 192**,** 99-108.

HALE, C. R., ZHAO, P., OLSON, S., DUFF, M. O., GRAVELEY, B. R., WELLS, L., et al. 2009. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell,* 139**,** 945-956.

HAMBLY, E., TÉTART, F., DESPLATS, C., WILSON, W. H., KRISCH, H. M. & MANN, N. H. 2001. A conserved genetic module that encodes the major virion components in both the coliphage T4 and the marine cyanophage S-PM2. *Proceedings of the National Academy of Sciences,* 98**,** 11411-11416.

HAN, M. K., HUANG, Y. J., LIPUMA, J. J., BOUSHEY, H. A., BOUCHER, R. C., COOKSON, W. O., et al. 2012. Significance of the microbiome in obstructive lung disease. *Thorax,* 67**,** 456-463.

HANCOCK, R. 1987. Role of porins in outer membrane permeability. *Journal of bacteriology,* 169**,** 929.

HANCOCK, R. & SPEERT, D. 1996. Antibiotics for Pseudomonas and related infections. *Cystic fibrosis current topics,* 3**,** 245-266.

HANCOCK, R. E. & KNOWLES, D. 1998. Are we approaching the end of the antibiotic era? *Current opinion in Microbiology,* 1**,** 493-494.

HANCOCK, R. E. & SPEERT, D. P. 2000. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. *Drug resistance updates,* 3**,** 247-255.

HANKIN, E. 1896. L'action bactericide des eaux de la Jumna et du Gange sur le vibrion du cholera. *Ann. Inst. Pasteur,* 10**,** 511.

HARDER, K. J., NIKAIDO, H. & MATSUHASHI, M. 1981. Mutants of Escherichia coli That Are Resistant to Certain Beta-Lactam Compounds Lack the ompF Porin. *Antimicrobial Agents and Chemotherapy,* 20**,** 549-552.

HARKNESS, R. E. & ÖLSCHLÄGER, T. 1991. The biology of colicin M. *FEMS microbiology reviews,* 88**,** 27-42.

HART, C. A. & WINSTANLEY, C. 2002. Persistent and aggressive bacteria in the lungs of cystic fibrosis children. *British medical bulletin,* 61**,** 81-96.

HATFULT, G. F. & SARKIS, G. J. 1993. DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. *Molecular microbiology,* 7**,** 395-405.

HATTMAN, S., WILKINSON, J., SWINTON, D., SCHLAGMAN, S., MACDONALD, P. & MOSIG, G. 1985. Common evolutionary origin of the phage T4 dam and host Escherichia coli dam DNA-adenine methyltransferase genes. *Journal of bacteriology,* 164**,** 932-937.

HATZINGER, P. B., PALMER, P., SMITH, R. L., PEÑARRIETA, C. T. & YOSHINARI, T. 2003. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. *Journal of microbiological methods,* 52**,** 47-58.

HAURWITZ, R. E., JINEK, M., WIEDENHEFT, B., ZHOU, K. & DOUDNA, J. A. 2010. Sequence-and structure-specific RNA processing by a CRISPR endonuclease. *Science,* 329**,** 1355-1358.

HAUSER, A. R. 2009. The type III secretion system of Pseudomonas aeruginosa: infection by injection. *Nat Rev Microbiol,* 7**,** 654-65.

HAUSER, A. R. 2011. Pseudomonas aeruginosa: So Many Virulence Factors, So Little Time. *Critical Care Medicine,* 39**,** 2193-2194.

HAUSER, A. R., JAIN, M., BAR-MEIR, M. & MCCOLLEY, S. A. 2011. Clinical significance of microbial infection and adaptation in cystic fibrosis. *Clinical microbiology reviews,* 24**,** 29-70.

HAUSMANN, R. & GOLD, M. 1966. The Enzymatic Methylation of Ribonucleic Acid and Deoxyribonucleic Acid IX. DEOXYRIBONUCLEIC ACID METHYLASE IN

BACTERIOPHAGE-INFECTED ESCHERICHIA COLI. *Journal of Biological Chemistry,* 241**,** 1985-1994.

HÄUßLER, S. 2004. Biofilm formation by the small colony variant phenotype of Pseudomonas aeruginosa. *Environmental microbiology,* 6**,** 546-551.

HÄUßLER, S., TÜMMLER, B., WEIßBRODT, H., ROHDE, M. & STEINMETZ, I. 1999. Small-colony variants of Pseudomonas aeruginosa in cystic fibrosis. *Clinical infectious diseases,* 29**,** 621-625.

HAUSSLER, S., ZIEGLER, I., LOTTEL, A., VON GOTZ, F., ROHDE, M., WEHMHOHNER, D., et al. 2003. Highly adherent small-colony variants of Pseudomonas aeruginosa in cystic fibrosis lung infection. *J Med Microbiol,* 52**,** 295-301.

HAYASHI, H., SAKAMOTO, M. & BENNO, Y. 2002. Phylogenetic Analysis of the Human Gut Microbiota Using 16S rDNA Clone Libraries and Strictly Anaerobic Culture-Based Methods. *Microbiology and Immunology,* 46**,** 535-548.

HAYASHI, T., BABA, T., MATSUMOTO, H. & TERAWAKI, Y. 1990. Phage-conversion of cytotoxin production in Pseudomonas aeruginosa. *Molecular microbiology,* 4**,** 1703-1709.

HAYASHI, T., MAKINO, K., OHNISHI, M., KUROKAWA, K., ISHII, K., YOKOYAMA, K., et al. 2001. Complete genome sequence of enterohemorrhagic Eschelichia coli O157: H7 and genomic comparison with a laboratory strain K-12. *DNA research,* 8**,** 11-22.

HEMPHILL, H. E. & WHITELEY, H. 1975. Bacteriophages of Bacillus subtilis. *Bacteriological reviews,* 39**,** 257.

HENDERSON, I. R., OWEN, P. & NATARO, J. P. 1999. Molecular switches—the ON and OFF of bacterial phase variation. *Molecular microbiology,* 33**,** 919-932.

HENDRIX, R. 2009. Jumbo bacteriophages. *Lesser Known Large dsDNA Viruses.* Springer.

HENDRIX, R. W., LAWRENCE, J. G., HATFULL, G. F. & CASJENS, S. 2000. The origins and ongoing evolution of viruses. *Trends in microbiology,* 8**,** 504-508.

HENDRIX, R. W., SMITH, M. C. M., BURNS, R. N., FORD, M. E. & HATFULL, G. F. 1999. Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage. *Proceedings of the National Academy of Sciences,* 96**,** 2192-2197.

HENGGE-ARONIS, R. 1993. Survival of hunger and stress: the role of rpoS in early stationary phase gene regulation in E. coli. *Cell,* 72**,** 165-168.

HENGGE-ARONIS, R. 2002. Signal transduction and regulatory mechanisms involved in control of the σS (RpoS) subunit of RNA polymerase. *Microbiology and Molecular Biology Reviews,* 66**,** 373-395.

HENRICHSEN, J. 1983. Twitching motility. *Annual Reviews in Microbiology,* 37**,** 81-93.

HEO, Y.-J., LEE, Y.-R., JUNG, H.-H., LEE, J., KO, G. & CHO, Y.-H. 2009. Antibacterial Efficacy of Phages against Pseudomonas aeruginosa Infections in Mice and Drosophila melanogaster. *Antimicrobial Agents and Chemotherapy,* 53**,** 2469-2474.

HERNÁNDEZ-SÁNCHEZ, J., BAUTISTA-SANTOS, A., FERNÁNDEZ, L., BERMÚDEZ-CRUZ, R. M., UC-MASS, A., MARTÍNEZ-PEÑAFIEL, E., et al. 2008. Analysis of some phenotypic traits of feces-borne temperate lambdoid bacteriophages from different immunity groups: a high incidence of cor+, FhuA-dependent phages. *Archives of virology,* 153**,** 1271-1280.

HERSKOWITZ, I. & HAGEN, D. 1980. The lysis-lysogeny decision of phage lambda: explicit programming and responsiveness. *Annu Rev Genet,* 14**,** 399-445.

HEURLIER, K., DÉNERVAUD, V., HAENNI, M., GUY, L., KRISHNAPILLAI, V. & HAAS, D. 2005. Quorum-Sensing-Negative (lasR) Mutants of Pseudomonas aeruginosa Avoid Cell Lysis and Death. *Journal of Bacteriology,* 187**,** 4875-4883.

HEYDER, J. 2004. Deposition of inhaled particles in the human respiratory tract and consequences for regional targeting in respiratory drug delivery. *Proceedings of the American Thoracic Society,* 1**,** 315-320.

HIGENBOTTAM, T., COCHRANE, G., CLARK, T., TURNER, D., MILLIS, R. & SEYMOUR, W. 1980. Bronchial disease in ulcerative colitis. *Thorax,* 35**,** 581-585.

HILL, D., SHORT, N., PERHAM, R. & PETERSEN, G. 1991. DNA sequence of the filamentous bacteriophage Pf1. *Journal of molecular biology,* 218**,** 349-364.

HO, C. S., LAM, C. W. K., CHAN, M. H. M., CHEUNG, R. C. K., LAW, L. K., LIT, L. C. W., et al. 2003. Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. *The Clinical Biochemist Reviews,* 24**,** 3-12.

HO, P.-L., CHAN, K.-N., IP, M. S., LAM, W.-K., HO, C.-S., YUEN, K.-Y., et al. 1998. The effect of Pseudomonas aeruginosa infection on clinical parameters in steady-state bronchiectasis. *CHEST Journal,* 114**,** 1594-1598.

HOFER, B., RUGE, M. & DREISEIKELMANN, B. 1995. The superinfection exclusion gene (sieA) of bacteriophage P22: identification and overexpression of the gene and localization of the gene product. *Journal of bacteriology,* 177**,** 3080-3086.

HOFFMANN, N., RASMUSSEN, T. B., JENSEN, P., STUB, C., HENTZER, M., MOLIN, S., et al. 2005. Novel mouse model of chronic Pseudomonas aeruginosa lung infection mimicking cystic fibrosis. *Infection and immunity,* 73**,** 2504-2514.

HOGARDT, M. & HEESEMANN, J. 2010. Adaptation of Pseudomonas aeruginosa during persistence in the cystic fibrosis lung. *International Journal of Medical Microbiology,* 300**,** 557-562.

HOIBY, N. 2011. Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis. *BMC medicine,* 9**,** 32.

HØIBY, N., KROGH JOHANSEN, H., MOSER, C., SONG, Z., CIOFU, O. & KHARAZMI, A. 2001. Pseudomonas aeruginosa and the in vitroand in vivo biofilm mode of growth. *Microbes and Infection,* 3**,** 23-35.

HOIDAL, J. R. 2001. Reactive Oxygen Species and Cell Signaling. *American Journal of Respiratory Cell and Molecular Biology,* 25**,** 661-663.

HOLGUÍN, A. V., RANGEL, G., CLAVIJO, V., PRADA, C., MANTILLA, M., GOMEZ, M. C., et al. 2015. Phage ΦPan70, a Putative Temperate Phage, Controls Pseudomonas aeruginosa in Planktonic, Biofilm and Burn Mouse Model Assays. *Viruses,* 7**,** 4602-4623.

HOLLOWAY, B. W., EGAN, J. B. & MONK, M. 1960. LYSOGENY IN PSEUDOMONAS AERUGINOSA[ast]. *Aust J Exp Biol Med,* 38**,** 321-330.

HOLMÉN, J. M., KARLSSON, N. G., ABDULLAH, L. H., RANDELL, S. H., SHEEHAN, J. K., HANSSON, G. C., et al. 2004. Mucins and their O-Glycans from human bronchial epithelial cell cultures. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 287**,** L824-L834.

HOLMES, R. K. 2000. Biology and molecular epidemiology of diphtheria toxin and the tox gene. *Journal of Infectious Diseases,* 181**,** S156-S167.

HONRAET, K., GOETGHEBEUR, E. & NELIS, H. J. 2005. Comparison of three assays for the quantification of Candida biomass in suspension and CDC reactor grown biofilms. *Journal of microbiological methods,* 63**,** 287-295.

HORDVIK, N. L., KÖNIG, P., HAMORY, B., COOPERSTOCK, M., KREUTZ, C., GAYER, D., et al. 1989. Effects of acute viral respiratory tract infections in patients with cystic fibrosis. *Pediatric pulmonology,* 7**,** 217-222.

HOUWINK, A. & VAN ITERSON, W. 1950. Electron microscopical observations on bacterial cytology II. A study of flagellation. *Biochimica et biophysica acta,* 5**,** 10-44.

HUECK, C. J. 1998. Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants. *Microbiology and Molecular Biology Reviews,* 62**,** 379-433.

HUSE, H. K., KWON, T., ZLOSNIK, J. E., SPEERT, D. P., MARCOTTE, E. M. & WHITELEY, M. 2013. Pseudomonas aeruginosa enhances production of a non-alginate exopolysaccharide during long-term colonization of the cystic fibrosis lung.

HUSON, D. H., AUCH, A. F., QI, J. & SCHUSTER, S. C. 2007. MEGAN analysis of metagenomic data. *Genome research,* 17**,** 377-386.

HUTABARAT, R. M., UNADKAT, J. D., KUSHMERICK, P., AITKEN, M. L., SLATTERY, J. T. & SMITH, A. L. 1991a. Disposition of drugs in cystic fibrosis. III. Acetaminophen. *Clinical Pharmacology & Therapeutics,* 50**,** 695-701.

HUTABARAT, R. M., UNADKAT, J. D., SAHAJWALLA, C., MCNAMARA, S., RAMSEY, B. & SMITH, A. L. 1991b. Disposition of drugs in cystic fibrosis. I. Sulfamethoxazole and trimethoprim. *Clinical Pharmacology & Therapeutics,* 49**,** 402-409.

HWANG, T. C. & SHEPPARD, D. N. 2009. Gating of the CFTR Cl− channel by ATP-driven nucleotide-binding domain dimerisation. *The Journal of physiology,* 587**,** 2151-2161.

IIDA, S., STREIFF, M. B., BICKLE, T. A. & ARBER, W. 1987. Two DNA antirestriction systems of bacteriophage P1, darA, and darB: characterization of darA- phages. *Virology,* 157**,** 156-66.

INOUYE, S., SUNSHINE, M. G., SIX, E. W. & INOUYE, M. 1991. Retronphage ΦR73: An E. coli phage that contains a retroelement and integrates into a tRNA gene. *Science,* 252**,** 969-971.

ISHINO, Y., SHINAGAWA, H., MAKINO, K., AMEMURA, M. & NAKATA, A. 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of bacteriology,* 169**,** 5429-5433.

ISLES, A., MACLUSKY, I., COREY, M., GOLD, R., PROBER, C., FLEMING, P., et al. 1984. Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. *The Journal of pediatrics,* 104**,** 206-210.

JACKSON, K. D., STARKEY, M., KREMER, S., PARSEK, M. R. & WOZNIAK, D. J. 2004. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for Pseudomonas aeruginosa PAO1 biofilm formation. *Journal of bacteriology,* 186**,** 4466-4475.

JACOBSON, A. 1972. Role of F Pili in the Penetration of Bacteriophage fl. *Journal of Virology,* 10**,** 835-843.

JAMES, C. E., DAVIES, E. V., FOTHERGILL, J. L., WALSHAW, M. J., BEALE, C. M., BROCKHURST, M. A., et al. 2015. Lytic activity by temperate phages of Pseudomonas aeruginosa in long-term cystic fibrosis chronic lung infections. *The ISME journal,* 9**,** 1391-1398.

JAMES, C. E., STANLEY, K. N., ALLISON, H. E., FLINT, H. J., STEWART, C. S., SHARP, R. J., et al. 2001. Lytic and Lysogenic Infection of DiverseEscherichia coli and Shigella Strains with a Verocytotoxigenic Bacteriophage. *Applied and environmental microbiology,* 67**,** 4335-4337.

JAPONI, A., FARSHAD, S. & ALBORZI, A. 2009. Pseudomonas aeruginosa: Burn Infection, Treatment and Antibacterial Resistance. *Iran Red Crescent Med J,* 11**,** 244-253.

JINEK, M., CHYLINSKI, K., FONFARA, I., HAUER, M., DOUDNA, J. A. & CHARPENTIER, E. 2012. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science,* 337**,** 816-821.

JOHNSON, K., PARKER, M. & LORY, S. 1986. Nucleotide sequence and transcriptional initiation site of two Pseudomonas aeruginosa pilin genes. *Journal of Biological Chemistry,* 261**,** 15703-15708.

JONES, A. M., GOVAN, J. R., DOHERTY, C. J., DODD, M. E., ISALSKA, B. J., STANBRIDGE, T. N., et al. 2001. Spread of a multiresistant strain of Pseudomonas aeruginosa in an adult cystic fibrosis clinic. *Lancet,* 358**,** 557-8.

JONES, S., YU, B., BAINTON, N. A., BIRDSALL, M., BYCROFT, B., CHHABRA, S., et al. 1993. The lux autoinducer regulates the production of exoenzyme virulence determinants in Erwinia carotovora and Pseudomonas aeruginosa. *The EMBO journal,* 12**,** 2477.

JØRGENSEN, F., BALLY, M., CHAPON-HERVE, V., MICHEL, G., LAZDUNSKI, A., WILLIAMS, P., et al. 1999. RpoS-dependent stress tolerance in Pseudomonas aeruginosa. *Microbiology,* 145**,** 835-844.

JOSEPHS, J. L. & SANDERS, M. 2004. Creation and comparison of MS/MS spectral libraries using quadrupole ion trap and triple-quadrupole mass spectrometers. *Rapid communications in mass spectrometry,* 18**,** 743-759.

JUHALA, R. J., FORD, M. E., DUDA, R. L., YOULTON, A., HATFULL, G. F. & HENDRIX, R. W. 2000. Genomic sequences of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid bacteriophages. *Journal of molecular biology,* 299**,** 27-51.

JUSKO, W. J., MOSOVICH, L. L., GERBRACHT, L. M., MATTAR, M. E. & YAFFE, S. J. 1975. Enhanced renal excretion of dicloxacillin in patients with cystic fibrosis. *Pediatrics,* 56**,** 1038-1044.

KAINULAINEN, L., NIKOSKELAINEN, J., VUORINEN, T., TEVOLA, K., LIIPPO, K. & RUUSKANEN, O. 1999. Viruses and Bacteria in Bronchial Samples from Patients with Primary Hypogammaglobulinemia. *American Journal of Respiratory and Critical Care Medicine,* 159**,** 1199-1204.

KAISER, A. D. & JACOB, F. 1957. Recombination between related temperate bacteriophages and the genetic control of immunity and prophage localization. *Virology,* 4**,** 509-521.

KALUZNY, K., ABEYRATHNE, P. D. & LAM, J. S. 2007. Coexistence of Two Distinct Versions of O-Antigen Polymerase, Wzy-Alpha and Wzy-Beta, in Pseudomonas aeruginosa Serogroup O2 and Their Contributions to Cell Surface Diversity. *Journal of Bacteriology,* 189**,** 4141-4152.

KAN, T. & HIDEO, T. 1994. Cloning, analysis and expression of an rpoS homologue gene from Pseudomonas aeruginosa PAO1. *Gene,* 150**,** 81-85.

KARAOLIS, D. K., SOMARA, S., MANEVAL, D. R., JR., JOHNSON, J. A. & KAPER, J. B. 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature,* 399**,** 375-9.

KENNAN, R. M., DHUNGYEL, O. P., WHITTINGTON, R. J., EGERTON, J. R. & ROOD, J. I. 2001. The Type IV Fimbrial Subunit Gene (fimA) ofDichelobacter nodosus Is Essential for Virulence, Protease Secretion, and Natural Competence. *Journal of bacteriology,* 183**,** 4451-4458.

KENZAKA, T., TANI, K. & NASU, M. 2010. High-frequency phage-mediated gene transfer in freshwater environments determined at single-cell level. *The ISME journal,* 4**,** 648-659.

KIEWITZ, C. & TÜMMLER, B. 2000. Sequence diversity of Pseudomonas aeruginosa: impact on population structure and genome evolution. *Journal of bacteriology,* 182**,** 3125-3135.

KIM, P. E., MUSHER, D. M., GLEZEN, W. P., BARRADAS, M. C. R., NAHM, W. K. & WRIGHT, C. E. 1996. Association of Invasive Pneumococcal Disease with Season, Atmospheric Conditions, Air Pollution, and the Isolation of Respiratory Viruses. *Clinical Infectious Diseases,* 22**,** 100-106.

KIM, S., RAHMAN, M., SEOL, S. Y., YOON, S. S. & KIM, J. 2012. Pseudomonas aeruginosa bacteriophage PA1Ø requires type IV pili for infection and shows broad bactericidal and biofilm removal activities. *Applied and environmental microbiology,* 78**,** 6380-6385.

KING, W., COLLINS, E. & BARRETT, E. 1983. Frequencies of bacteriophage-resistant and slow acid-producing variants of Streptococcus cremoris. *Applied and environmental microbiology,* 45**,** 1481-1485.

KIPNIS, E., SAWA, T. & WIENER-KRONISH, J. 2006. Targeting mechanisms of Pseudomonas aeruginosa pathogenesis. *Médecine et Maladies Infectieuses,* 36**,** 78-91.

KNOWLES, M. R. & BOUCHER, R. C. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. *The Journal of clinical investigation,* 109**,** 571-577.

KOBAYASHI, I. 2001. Behavior of restriction–modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic acids research,* 29**,** 3742-3756.

KÖHLER, T., KOK, M., MICHEA-HAMZEHPOUR, M., PLESIAT, P., GOTOH, N., NISHINO, T., et al. 1996. Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy,* 40**,** 2288-2290.

KOLLEF, M. H. 2001. Is there a role for antibiotic cycling in the intensive care unit? *Critical care medicine,* 29**,** N135-N142.

KORPPI, M., LEINONEN, M., KOSKELA, M., MÄKELÄ, P. H. & LAUNIALA, K. 1989. Bacterial coinfection in children hospitalized with respiratory syncytial virus infections. *The Pediatric infectious disease journal,* 8**,** 687-691.

KOUCHAK, M., AMERI, A., NASERI, B. & KARGAR BOLDAJI, S. 2014. Chitosan and polyvinyl alcohol composite films containing nitrofurazone: preparation and evaluation. *Iranian Journal of Basic Medical Sciences,* 17**,** 14-20.

KOURILSKY, P. 1973. Lysogenization by bacteriophage lambda. *Molecular and General Genetics MGG,* 122**,** 183-195.

KROPINSKI, A. M. 2000. Sequence of the Genome of the Temperate, Serotype-Converting, Pseudomonas aeruginosa Bacteriophage D3. *Journal of Bacteriology,* 182**,** 6066-6074.

KRÜGER, D. & BICKLE, T. A. 1983. Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. *Microbiological reviews,* 47**,** 345.

KRYLOV, V., SMIRNOVA, T., REBENTISH, B. & MINENKOVA, I. 1977. [Stucture of PhiKZ bacteriophage particles]. *Voprosy virusologii***,** 568-571.

KUHN, D., BALKIS, M., CHANDRA, J., MUKHERJEE, P. & GHANNOUM, M. 2003. Uses and limitations of the XTT assay in studies of Candida growth and metabolism. *Journal of Clinical Microbiology,* 41**,** 506-508.

KUHN, D. M., CHANDRA, J., MUKHERJEE, P. K. & GHANNOUM, M. A. 2002a. Comparison of Biofilms Formed by Candida albicans and Candida parapsilosis on Bioprosthetic Surfaces. *Infection and Immunity,* 70**,** 878-888.

KUHN, D. M., GEORGE, T., CHANDRA, J., MUKHERJEE, P. K. & GHANNOUM, M. A. 2002b. Antifungal Susceptibility of Candida Biofilms: Unique Efficacy of Amphotericin B Lipid Formulations and Echinocandins. *Antimicrobial Agents and Chemotherapy,* 46**,** 1773-1780.

KÜHNLEIN, U. & ARBER, W. 1972. Host specificity of DNA produced by Escherichia coli: XV. The role of nucleotide methylation in in vitro B-specific modification. *Journal of molecular biology,* 63**,** 9-19.

KULAKOV, L. A., KSENZENKO, V. N., SHLYAPNIKOV, M. G., KOCHETKOV, V. V., DEL CASALE, A., ALLEN, C. C., et al. 2009. Genomes of "phiKMV-like viruses" of Pseudomonas aeruginosa contain localized single-strand interruptions. *Virology,* 391**,** 1-4.

KUS, J. V., TULLIS, E., CVITKOVITCH, D. G. & BURROWS, L. L. 2004. Significant differences in type IV pilin allele distribution among Pseudomonas aeruginosa isolates from cystic fibrosis (CF) versus non-CF patients. *Microbiology,* 150**,** 1315-1326.

LAARMAN, A. J., BARDOEL, B. W., RUYKEN, M., FERNIE, J., MILDER, F. J., VAN STRIJP, J. A. G., et al. 2012. Pseudomonas aeruginosa Alkaline Protease Blocks Complement Activation via the Classical and Lectin Pathways. *The Journal of Immunology,* 188**,** 386-393.

LABRIE, S. J., SAMSON, J. E. & MOINEAU, S. 2010a. Bacteriophage resistance mechanisms. *Nature reviews. Microbiology,* 8**,** 317-27.

LABRIE, S. J., SAMSON, J. E. & MOINEAU, S. 2010b. Bacteriophage resistance mechanisms. *Nature Reviews Microbiology,* 8**,** 317-327.

LACEY, R. 1975. Antibiotic resistance plasmids of Staphylococcus aureus and their clinical importance. *Bacteriological Reviews,* 39**,** 1.

LADHANI, S., JOANNOU, C. L., LOCHRIE, D. P., EVANS, R. W. & POSTON, S. M. 1999. Clinical, Microbial, and Biochemical Aspects of the Exfoliative Toxins Causing Staphylococcal Scalded-Skin Syndrome. *Clinical Microbiology Reviews,* 12**,** 224-242.

LAENNEC, R. 1962. A treatise on the disease of the chest. Forbes J, trans. New York: Library of the New York Academy of Medicine. Hafner Publishing.

LAFAYETTE, S. L., HOULE, D., BEAUDOIN, T., WOJEWODKA, G., RADZIOCH, D., HOFFMAN, L. R., et al. 2015. Cystic fibrosis–adapted Pseudomonas aeruginosa quorum sensing lasR mutants cause hyperinflammatory responses. *Science Advances,* 1**,** e1500199.

LAMMENS, E., CEYSSENS, P.-J., VOET, M., HERTVELDT, K., LAVIGNE, R. & VOLCKAERT, G. 2009. Representational Difference Analysis (RDA) of bacteriophage genomes. *Journal of microbiological methods,* 77**,** 207-213.

LANGE, R. & HENGGE-ARONIS, R. 1994. The cellular concentration of the sigma S subunit of RNA polymerase in Escherichia coli is controlled at the levels of transcription, translation, and protein stability. *Genes & development,* 8**,** 1600-1612.

LAPLANTE, K. L. & WOODMANSEE, S. 2009. Activities of Daptomycin and Vancomycin Alone and in Combination with Rifampin and Gentamicin against Biofilm-Forming Methicillin-Resistant Staphylococcus aureus Isolates in an Experimental Model of Endocarditis. *Antimicrobial Agents and Chemotherapy,* 53**,** 3880-3886.

LATIFI, A., FOGLINO, M., TANAKA, K., WILLIAMS, P. & LAZDUNSKI, A. 1996. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Molecular microbiology,* 21**,** 1137-1146.

LAUTROP, H. 1961. Bacterium anitratum transferred to the genus Cytophaga*. *International Journal of Systematic and Evolutionary Microbiology,* 11**,** 107-108.

LAWRENCE, J. G. & OCHMAN, H. 1998. Molecular archaeology of the Escherichia coli genome. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 9413-7.

LAZAREVIC, V., WHITESON, K., HUSE, S., HERNANDEZ, D., FARINELLI, L., ØSTERÅS, M., et al. 2009. Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. *Journal of Microbiological Methods,* 79**,** 266-271.

LEDERBERG, E. M. LYSOGENICITY IN E-COLI K-12. Genetics, 1951. 428 EAST PRESTON ST, BALTIMORE, MD 21202, 560-560.

LEE, A., MAO, W., WARREN, M. S., MISTRY, A., HOSHINO, K., OKUMURA, R., et al. 2000. Interplay between Efflux Pumps May Provide Either Additive or Multiplicative Effects on Drug Resistance. *Journal of Bacteriology,* 182**,** 3142-3150.

LEE, B., HAAGENSEN, J. A., CIOFU, O., ANDERSEN, J. B., HOIBY, N. & MOLIN, S. 2005a. Heterogeneity of biofilms formed by nonmucoid Pseudomonas aeruginosa isolates from patients with cystic fibrosis. *J Clin Microbiol,* 43**,** 5247-55.

LEE, B., HAAGENSEN, J. A., CIOFU, O., ANDERSEN, J. B., HØIBY, N. & MOLIN, S. 2005b. Heterogeneity of biofilms formed by nonmucoid Pseudomonas aeruginosa isolates from patients with cystic fibrosis. *Journal of clinical microbiology,* 43**,** 5247-5255.

LEE, C. Y. & IANDOLO, J. J. 1985. Mechanism of bacteriophage conversion of lipase activity in Staphylococcus aureus. *Journal of bacteriology,* 164**,** 288-293.

LEHNHERR, H. & YARMOLINSKY, M. B. 1995. Addiction protein phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 3274-3277.

LENSKI, R. E. & LEVIN, B. R. 1985. Constraints on the coevolution of bacteria and virulent phage: a model, some experiments, and predictions for natural communities. *American Naturalist***,** 585-602.

LEONE, I., CHIRILLO, M. G., RASO, T., ZUCCA, M. & SAVOIA, D. 2008. Phenotypic and genotypic characterization of Pseudomonas aeruginosa from cystic fibrosis patients. *Eur J Clin Microbiol Infect Dis,* 27**,** 1093-9.

LEONG, D. & MURPHY, J. R. 1985. Characterization of the diphtheria tox transcript in Corynebacterium diphtheriae and Escherichia coli. *Journal of Bacteriology,* 163**,** 1114-1119.

LEPLAE, R., GEERAERTS, D., HALLEZ, R., GUGLIELMINI, J., DREZE, P. & VAN MELDEREN, L. 2011. Diversity of bacterial type II toxin-antitoxin systems: a comprehensive search and functional analysis of novel families. *Nucleic Acids Res,* 39**,** 5513-25.

LEVY, J., SMITH, A. L., KOUP, J. R., WILLIAMS-WARREN, J. & RAMSEY, B. 1984. Disposition of tobramycin in patients with cystic fibrosis: a prospective controlled study. *The Journal of pediatrics,* 105**,** 117-124.

LI, X.-Z., NIKAIDO, H. & POOLE, K. 1995. Role of mexA-mexB-oprM in antibiotic efflux in Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy,* 39**,** 1948-1953.

LI, X.-Z., ZHANG, L. & POOLE, K. 2000. Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of Pseudomonas aeruginosa. *Journal of Antimicrobial Chemotherapy,* 45**,** 433-436.

LIEB, M. 1953. The establishment of lysogenicity in Escherichia coli. *Journal of bacteriology,* 65**,** 642.

LILLEENGEN, K. 1984. *Typing of Salmonella typhi murium by means of bacteriophage: an experimental bacteriologic study for the purpose of devising a phage-typing method to be used as an aid in epidemiologic and epizootologic investigations in outbreaks of typhimurium infection.* Haeggström.

LIM, Y. W., EVANGELISTA, J. S., SCHMIEDER, R., BAILEY, B., HAYNES, M., FURLAN, M., et al. 2014. Clinical Insights from Metagenomic Analysis of Sputum Samples from Patients with Cystic Fibrosis. *Journal of Clinical Microbiology,* 52**,** 425-437.

LIN, L., BITNER, R. & EDLIN, G. 1977. Increased reproductive fitness of Escherichia coli lambda lysogens. *Journal of virology,* 21**,** 554-559.

LIPUMA, J. J. 2010. The changing microbial epidemiology in cystic fibrosis. *Clinical microbiology reviews,* 23**,** 299-323.

LIVERMORE, D. M. 2002. Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? *Clinical infectious diseases,* 34**,** 634-640.

LU, M. & HENNING, U. 1989. The immunity (imm) gene of Escherichia coli bacteriophage T4. *Journal of virology,* 63**,** 3472-3478.

LU, M., STIERHOF, Y. & HENNING, U. 1993. Location and unusual membrane topology of the immunity protein of the Escherichia coli phage T4. *Journal of virology,* 67**,** 4905-4913.

LURIA, S. E. & ANDERSON, T. F. 1942. The identification and characterization of bacteriophages with the electron microscope. *Proceedings of the National Academy of Sciences of the United States of America,* 28**,** 127.

LURIA, S. E., DELBRÜCK, M. & ANDERSON, T. F. 1943. Electron microscope studies of bacterial viruses. *Journal of bacteriology,* 46**,** 57.

LYELL, A. 1956. Toxic epidermal necrolysis: an eruption resembling scalding of the skin. *British Journal of Dermatology,* 68**,** 355-361.

LYON, B. R. & SKURRAY, R. 1987. Antimicrobial resistance of Staphylococcus aureus: genetic basis. *Microbiological reviews,* 51**,** 88.

M BALCAO, V., G SANTOS, M., R MARTINS, P., V CHAUD, M., M DE OLIVEIRA JUNIOR, J., TUBINO, M., et al. 2013. Development and Characterization of a Gel Formulation Integrating Microencapsulated Nitrofurazone. *Current pharmaceutical biotechnology,* 14**,** 1036-1047.

MA, D., COOK, D. N., HEARST, J. E. & NIKAIDO, H. 1994. Efflux pumps and drug resistance in gram-negative bacteria. *Trends in microbiology,* 2**,** 489-493.

MA, L., CONOVER, M., LU, H., PARSEK, M. R., BAYLES, K. & WOZNIAK, D. J. 2009. Assembly and development of the Pseudomonas aeruginosa biofilm matrix. *PLoS Pathog,* 5**,** e1000354.

MAATALLAH, M., CHERIAA, J., BACKHROUF, A., IVERSEN, A., GRUNDMANN, H., DO, T., et al. 2011. Population Structure of <italic>Pseudomonas aeruginosa</italic> from Five Mediterranean Countries: Evidence for Frequent Recombination and Epidemic Occurrence of CC235. *PLoS ONE,* 6**,** e25617.

MACFARLANE, E. L., KWASNICKA, A. & HANCOCK, R. E. 2000. Role of Pseudomonas aeruginosa PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. *Microbiology,* 146**,** 2543-2554.

MAHADEVAN, S., SHAH, S. L., MARRIE, T. J. & SLUPSKY, C. M. 2008. Analysis of Metabolomic Data Using Support Vector Machines. *Analytical Chemistry,* 80**,** 7562-7570.

MAHENTHIRALINGAM, E., CAMPBELL, M. E. & SPEERT, D. P. 1994. Nonmotility and phagocytic resistance of Pseudomonas aeruginosa isolates from chronically colonized patients with cystic fibrosis. *Infection and Immunity,* 62**,** 596-605.

MAIER, R. & SOBERON-CHAVEZ, G. 2000. Pseudomonas aeruginosa rhamnolipids: biosynthesis and potential applications. *Applied Microbiology and Biotechnology,* 54**,** 625-633.

MAKAROVA, K. S., HAFT, D. H., BARRANGOU, R., BROUNS, S. J., CHARPENTIER, E., HORVATH, P., et al. 2011. Evolution and classification of the CRISPR–Cas systems. *Nature Reviews Microbiology,* 9**,** 467-477.

MAKAROVA, K. S., WOLF, Y. I. & KOONIN, E. V. 2013. Comparative genomics of defense systems in archaea and bacteria. *Nucleic Acids Research*.

MALONE, J. G., JAEGER, T., MANFREDI, P., DÖTSCH, A., BLANKA, A., BOS, R., et al. 2012. The YfiBNR signal transduction mechanism reveals novel targets for the evolution of persistent Pseudomonas aeruginosa in cystic fibrosis airways. *PLoS pathogens,* 8**,** e1002760.

MANICHANH, C., RIGOTTIER-GOIS, L., BONNAUD, E., GLOUX, K., PELLETIER, E., FRANGEUL, L., et al. 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut,* 55**,** 205-211.

MANN, M. & FENN, J. B. 1992. Electrospray mass spectrometry. *Mass Spectrometry.* Springer.

MANN, M., MENG, C. K. & FENN, J. B. 1989. Interpreting mass spectra of multiply charged ions. *Analytical Chemistry,* 61**,** 1702-1708.

MANOS, J., ARTHUR, J., ROSE, B., BELL, S., TINGPEJ, P., HU, H., et al. 2009. Gene expression characteristics of a cystic fibrosis epidemic strain of Pseudomonas aeruginosa during biofilm and planktonic growth. *FEMS microbiology letters,* 292**,** 107-114.

MANOS, J., ARTHUR, J., ROSE, B., TINGPEJ, P., FUNG, C., CURTIS, M., et al. 2008. Transcriptome analyses and biofilm-forming characteristics of a clonal Pseudomonas aeruginosa from the cystic fibrosis lung. *Journal of medical microbiology,* 57**,** 1454-1465.

MARKS, M. I. 1981. The pathogenesis and treatment of pulmonary infections in patients with cystic fibrosis. *J Pediatr,* 98**,** 173-9.

MARTIN, C., ICHOU, M. A., MASSICOT, P., GOUDEAU, A. & QUENTIN, R. 1995. Genetic diversity of Pseudomonas aeruginosa strains isolated from patients with cystic fibrosis revealed by restriction fragment length polymorphism of the rRNA gene region. *Journal of clinical microbiology,* 33**,** 1461-1466.

MARTINI, N., AGOSTINI, M., BARLOCCO, G., BOZZINI, L., CASTELLANI, L., MESSORI, A., et al. 1984. Serum and sputum concentrations of azlocillin, cefoperazone and ceftazidime in patients with cystic fibrosis. *Journal of Clinical Pharmacy and Therapeutics,* 9**,** 303-309.

MARVIG, R. L., SOMMER, L. M., MOLIN, S. & JOHANSEN, H. K. 2015. Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. *Nat Genet,* 47**,** 57-64.

MARVIN, D. A. & HOHN, B. 1969. Filamentous bacterial viruses. *Bacteriological Reviews,* 33**,** 172-209.

MASTERTON, R. G. 2005. Antibiotic cycling: more than it might seem? *Journal of Antimicrobial Chemotherapy,* 55**,** 1-5.

MASUDA, H., TAN, Q., AWANO, N., WU, K. P. & INOUYE, M. 2012. YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in Escherichia coli. *Molecular microbiology,* 84**,** 979-989.

MASUDA, N., GOTOH, N., ISHII, C., SAKAGAWA, E., OHYA, S. & NISHINO, T. 1999. Interplay between Chromosomal β-Lactamase and the MexAB-OprM Efflux System in Intrinsic Resistance to β-Lactams inPseudomonas aeruginosa. *Antimicrobial agents and chemotherapy,* 43**,** 400-402.

MATHEE, K., CIOFU, O., STERNBERG, C., LINDUM, P. W., CAMPBELL, J. I., JENSEN, P., et al. 1999. Mucoid conversion of Pseudomonas aeruginos by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology,* 145**,** 1349-1357.

MATSUI, H., GRUBB, B. R., TARRAN, R., RANDELL, S. H., GATZY, J. T., DAVIS, C. W., et al. 1998. Evidence for Periciliary Liquid Layer Depletion, Not Abnormal Ion Composition, in the Pathogenesis of Cystic Fibrosis Airways Disease. *Cell,* 95**,** 1005-1015.

MATSUSHIRO, A., SATO, K., MIYAMOTO, H., YAMAMURA, T. & HONDA, T. 1999. Induction of prophages of enterohemorrhagic Escherichia coli O157:H7 with norfloxacin. *Journal of bacteriology,* 181**,** 2257-60.

MATTICK, J. S. 2002. Type IV pili and twitching motility. *Annual Reviews in Microbiology,* 56**,** 289-314.

MAY, R. M. & ANDERSON, R. 1983. Epidemiology and genetics in the coevolution of parasites and hosts. *Proceedings of the Royal Society of London B: Biological Sciences,* 219**,** 281-313.

MCCALLUM, S. J., CORKILL, J., GALLAGHER, M., LEDSON, M. J., HART, C. A. & WALSHAW, M. J. 2001. Superinfection with a transmissible strain of Pseudomonas aeruginosa in adults with cystic fibrosis chronically colonised by P aeruginosa. *Lancet,* 358**,** 558-60.

MCCALLUM, S. J., GALLAGHER, M. J., CORKILL, J. E., HART, C. A., LEDSON, M. J. & WALSHAW, M. J. 2002. Spread of an epidemic Pseudomonas aeruginosa strain from a patient with cystic fibrosis (CF) to non-CF relatives. *Thorax,* 57**,** 559-60.

MCCLUSKEY, C., QUINN, J. & MCGRATH, J. 2005. An evaluation of three new-generation tetrazolium salts for the measurement of respiratory activity in activated sludge microorganisms. *Microbial ecology,* 49**,** 379-387.

MCGOWAN, J. E., JR. 1986. Minimizing antimicrobial resistance in hospital bacteria: can switching or cycling drugs help? *Infect Control,* 7**,** 573-6.

MCLENNAN, A., BATES, A., TURNER, P. & WHITE, M. 2012. *BIOS Instant Notes in Molecular Biology*, Taylor & Francis.

MCMAHON, M., SWINSON, D., SHETTAR, S., WOLSTENHOLME, R., CHATTOPADHYAY, C., SMITH, P., et al. 1993. Bronchiectasis and rheumatoid arthritis: a clinical study. *Annals of the rheumatic diseases,* 52**,** 776-779.

MCMENAMIN, J. D., ZACCONE, T. M., COENYE, T., VANDAMME, P. & LIPUMA, J. J. 2000. Misidentification of Burkholderia cepacia in US cystic fibrosis treatment centers: an analysis of 1,051 recent sputum isolates. *CHEST Journal,* 117**,** 1661-1665.

MCOSKER, C. C. & FITZPATRICK, P. M. 1994. Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. *J Antimicrob Chemother,* 33 Suppl A**,** 23-30.

MCSHAN, W. M., TANG, Y. F. & FERRETTI, J. J. 1997. Bacteriophage T12 of Streptococcus pyogenes integrates into the gene encoding a serine tRNA. *Molecular microbiology,* 23**,** 719-728.

MEDIAVILLA, J., JAIN, S., KRIAKOV, J., FORD, M. E., DUDA, R. L., JACOBS, W. R., et al. 2000. Genome organization and characterization of mycobacteriophage Bxb1. *Molecular microbiology,* 38**,** 955-970.

MELISH, M. E. & GLASGOW, L. A. 1970. The staphylococcal scalded-skin syndrome: development of an experimental model. *New England Journal of Medicine,* 282**,** 1114-1119.

MEYER, M. & KIRCHER, M. 2010. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harb Protoc,* 2010**,** t5448.

MEYER, M., STENZEL, U. & HOFREITER, M. 2008. Parallel tagged sequencing on the 454 platform. *Nature protocols,* 3**,** 267-278.

MICHALOWSKI, C. B. & LITTLE, J. W. 2005. Positive autoregulation of cI is a dispensable feature of the phage lambda gene regulatory circuitry. *J Bacteriol,* 187**,** 6430-42.

MILLER, M. B. & BASSLER, B. L. 2001. Quorum sensing in bacteria. *Annual Reviews in Microbiology,* 55**,** 165-199.

MINDICH, L., QIAO, X., QIAO, J., ONODERA, S., ROMANTSCHUK, M. & HOOGSTRATEN, D. 1999. Isolation of additional bacteriophages with genomes of segmented double-stranded RNA. *Journal of bacteriology,* 181**,** 4505-4508.

MIROLD, S., RABSCH, W., ROHDE, M., STENDER, S., TSCHÄPE, H., RÜSSMANN, H., et al. 1999. Isolation of a temperate bacteriophage encoding the type III effector

protein SopE from an epidemic Salmonella typhimurium strain. *Proceedings of the National Academy of Sciences,* 96**,** 9845-9850.

MODI, S. R., LEE, H. H., SPINA, C. S. & COLLINS, J. J. 2013. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature,* 499**,** 219-222.

MOHAN, K., FOTHERGILL, J., STORRAR, J., LEDSON, M., WINSTANLEY, C. & WALSHAW, M. 2008. Transmission of Pseudomonas aeruginosa epidemic strain from a patient with cystic fibrosis to a pet cat. *Thorax,* 63**,** 839-840.

MOLKANEN, T., TYYNELA, J., HELIN, J., KALKKINEN, N. & KUUSELA, P. 2002. Enhanced activation of bound plasminogen on Staphylococcus aureus by staphylokinase. *FEBS letters,* 517**,** 72-8.

MONIAUX, N., ESCANDE, F., PORCHET, N., AUBERT, J. & BATRA, S. 2001. Structural organization and classification of the human mucin genes. *Frontiers in bioscience: a journal and virtual library,* 6**,** D1192-206.

MORGAN, G. J., HATFULL, G. F., CASJENS, S. & HENDRIX, R. W. 2002. Bacteriophage Mu genome sequence: analysis and comparison with Mu-like prophages in< i> Haemophilus, Neisseria</i> and< i> Deinococcus</i>. *Journal of molecular biology,* 317**,** 337-359.

MORILLAS, H. N., ZARIWALA, M. & KNOWLES, M. R. 2007. Genetic causes of bronchiectasis: primary ciliary dyskinesia. *Respiration,* 74**,** 252-263.

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods,* 65**,** 55-63.

MOSS, J. E., CARDOZO, T. J., ZYCHLINSKY, A. & GROISMAN, E. A. 1999. The selC-associated SHI-2 pathogenicity island of Shigella flexneri. *Molecular microbiology,* 33**,** 74-83.

MOSS, W. J., BEERS, M. C., JOHNSON, E., NICHOLS, D. G., PERL, T. M., DICK, J. D., et al. 2002. Pilot study of antibiotic cycling in a pediatric intensive care unit. *Critical care medicine,* 30**,** 1877-1882.

MOUNEIMNÉ, H., ROBERT, J., JARLIER, V. & CAMBAU, E. 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy,* 43**,** 62-66.

MOWAT, E., PATERSON, S., FOTHERGILL, J. L., WRIGHT, E. A., LEDSON, M. J., WALSHAW, M. J., et al. 2011. Pseudomonas aeruginosa Population Diversity and Turnover in Cystic Fibrosis Chronic Infections. *American Journal of Respiratory and Critical Care Medicine,* 183**,** 1674-1679.

MURPHY, T. F. & SETHI, S. 1992. Bacterial Infection in Chronic Obstructive Pulmonary Disease. *American Review of Respiratory Disease,* 146**,** 1067-1083.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and environmental microbiology,* 59**,** 695-700.

NAKAE, T., NAKAJIMA, A., ONO, T., SAITO, K. & YONEYAMA, H. 1999. Resistance to β-Lactam Antibiotics inPseudomonas aeruginosa Due to Interplay between the MexAB-OprM Efflux Pump and β-Lactamase. *Antimicrobial agents and chemotherapy,* 43**,** 1301-1303.

NANDA, A. M., THORMANN, K. & FRUNZKE, J. 2015. Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. *Journal of bacteriology,* 197**,** 410-419.

NICOTRA, M. B., RIVERA, M., DALE, A. M., SHEPHERD, R. & CARTER, R. 1995. Clinical, pathophysiologic, and microbiologic characterization of bronchiectasis in an aging cohort. *CHEST Journal,* 108**,** 955-961.

NIEDERMAN, M. S. 1997. Is "Crop Rotation" of Antibiotics the Solution to a "Resistant" Problem in the ICU? *American Journal of Respiratory and Critical Care Medicine,* 156**,** 1029-1031.

NIESSEN, W. M. 2006. *Liquid chromatography-mass spectrometry*, CRC Press.

NIKAIDO, H. 1992. Porins and specific channels of bacterial outer membranes. *Molecular microbiology,* 6**,** 435-442.

NIKAIDO, H. 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Seminars in Cell & Developmental Biology,* 12**,** 215-223.

NIKAIDO, H. 2003. Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiology and Molecular Biology Reviews,* 67**,** 593-656.

NISHIMURA, S., TSURUMOTO, T., YONEKURA, A., ADACHI, K. & SHINDO, H. 2006. Antimicrobial susceptibility of Staphylococcus aureus and Staphylococcus epidermidis biofilms isolated from infected total hip arthroplasty cases. *J Orthop Sci,* 11**,** 46-50.

NNALUE, N. A., NEWTON, S. & STOCKER, B. A. 1990. Lysogenization of Salmonella choleraesuis by phage 14 increases average length of O-antigen chains, serum resistance and intraperitoneal mouse virulence. *Microbial pathogenesis,* 8**,** 393-402.

NNALUE, N. A. & STOCKER, B. 1986. Some galE mutants of Salmonella choleraesuis retain virulence. *Infection and immunity,* 54**,** 635-640.

NOVICK, R. P. 2000. Pathogenicity factors and their regulation. *Gram-positive pathogens. ASM Press, Washington, DC***,** 392-407.

NOVOTNY, C. P. & FIVES-TAYLOR, P. 1974. Retraction of F Pili. *Journal of Bacteriology,* 117**,** 1306-1311.

NUNN, D., BERGMAN, S. & LORY, S. 1990. Products of three accessory genes, pilB, pilC, and pilD, are required for biogenesis of Pseudomonas aeruginosa pili. *Journal of bacteriology,* 172**,** 2911-2919.

O'CARROLL, M., SYRMIS, M., WAINWRIGHT, C., GREER, R., MITCHELL, P., COULTER, C., et al. 2004. Clonal strains of Pseudomonas aeruginosa in paediatric and adult cystic fibrosis units. *European Respiratory Journal,* 24**,** 101-106.

O'TOOLE, G. A. & KOLTER, R. 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Molecular microbiology,* 30**,** 295-304.

OBREGÓN, V., GARCÍA, J. L., GARCÍA, E., LÓPEZ, R. & GARCÍA, P. 2003. Genome Organization and Molecular Analysis of the Temperate Bacteriophage MM1 of Streptococcus pneumoniae. *Journal of Bacteriology,* 185**,** 2362-2368.

OCHMAN, H., LAWRENCE, J. G. & GROISMAN, E. A. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature,* 405**,** 299-304.

OCHSNER, U. A., KOCH, A. K., FIECHTER, A. & REISER, J. 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. *Journal of Bacteriology,* 176**,** 2044-2054.

OCHSNER, U. A. & REISER, J. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences,* 92**,** 6424-6428.

OHNISHI, M., KUROKAWA, K. & HAYASHI, T. 2001. Diversification of Escherichia coli genomes: are bacteriophages the major contributors? *Trends in microbiology,* 9**,** 481-5.

OJENIYI, B., BIRCH-ANDERSEN, A., MANSA, B., ROSDAHL, V. T. & HOIBY, N. 1991. Morphology of Pseudomonas aeruginosa phages from the sputum of cystic fibrosis patients and from the phage typing set. An electron microscopy study. *Apmis,* 99**,** 925-30.

OLIVER, A., CANTÓN, R., CAMPO, P., BAQUERO, F. & BLÁZQUEZ, J. 2000. High Frequency of Hypermutable Pseudomonas aeruginosa in Cystic Fibrosis Lung Infection. *Science,* 288**,** 1251-1253.

OLSEN, R. H. & SHIPLEY, P. 1973. Host range and properties of the Pseudomonas aeruginosa R factor R1822. *Journal of Bacteriology,* 113**,** 772-780.

OLSZAK, T., ZARNOWIEC, P., KACA, W., DANIS-WLODARCZYK, K., AUGUSTYNIAK, D., DREVINEK, P., et al. 2015. In vitro and in vivo antibacterial activity of environmental bacteriophages against Pseudomonas aeruginosa strains from cystic fibrosis patients. *Applied microbiology and biotechnology,* 99**,** 6021-6033.

ONG, E., ELLIS, M., WEBB, A., NEAL, K., DODD, M., CAUL, E., et al. 1989. Infective respiratory exacerbations in young adults with cystic fibrosis: role of viruses and atypical microorganisms. *Thorax,* 44**,** 739-742.

ORTMANN, A. C. & SUTTLE, C. A. 2005. High abundances of viruses in a deep-sea hydrothermal vent system indicates viral mediated microbial mortality. *Deep Sea Research Part I: Oceanographic Research Papers,* 52**,** 1515-1527.

PACE, N. R., STAHL, D. A., LANE, D. J. & OLSEN, G. J. 1985. Analyzing natural microbial populations by rRNA sequences. *ASM American Society for Microbiology News,* 51**,** 4-12.

PADERA, R. F. 2006. Infection in ventricular assist devices: the role of biofilm. *Cardiovasc Pathol,* 15**,** 264-70.

PALMER, K. L., AYE, L. M. & WHITELEY, M. 2007. Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum. *Journal of bacteriology,* 189**,** 8079-8087.

PALMER, K. L., MASHBURN, L. M., SINGH, P. K. & WHITELEY, M. 2005. Cystic fibrosis sputum supports growth and cues key aspects of Pseudomonas aeruginosa physiology. *Journal of bacteriology,* 187**,** 5267-5277.

PALOMINO, J.-C., MARTIN, A., CAMACHO, M., GUERRA, H., SWINGS, J. & PORTAELS, F. 2002. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy,* 46**,** 2720-2722.

PANG, J. A., CHENG, A., CHAN, H., POON, D. & FRENCH, G. 1989. The bacteriology of bronchiectasis in Hong Kong investigated by protected catheter brush and bronchoalveolar lavage. *Am Rev Respir Dis,* 139**,** 14-17.

PARKER, M., TOMLINSON, A. & WILLIAMS, R. 1955. Impetigo contagiosa. The association of certain types of Staphylococcus aureus and of Streptococcus pyogenes with superficial skin infections. *Journal of Hygiene,* 53**,** 458-473.

PARSONS, Y. N., PANAGEA, S., SMART, C. H., WALSHAW, M. J., HART, C. A. & WINSTANLEY, C. 2002. Use of subtractive hybridization to identify a diagnostic probe for a cystic fibrosis epidemic strain of Pseudomonas aeruginosa. *Journal of clinical microbiology,* 40**,** 4607-4611.

PASLOSKE, B. L., FINLAY, B. B. & PARANCHYCH, W. 1985. Cloning and sequencing of the Pseudomonas aeruginosa PAK pilin gene. *FEBS letters,* 183**,** 413-416.

PASSADOR, L., COOK, J. M., GAMBELLO, M. J., RUST, L. & IGLEWSKI, B. H. 1993. Expression of Pseudomonas aeruginosa virulence genes requires cell-to-cell communication. *Science,* 260**,** 1127-1130.

PASTEUR, M., HELLIWELL, S., HOUGHTON, S., WEBB, S., FOWERAKER, J., COULDEN, R., et al. 2000. An Investigation into Causative Factors in Patients with Bronchiectasis. *American Journal of Respiratory and Critical Care Medicine,* 162**,** 1277-1284.

PAUL, J. H. 1999. Microbial gene transfer: an ecological perspective. *J Mol Microbiol Biotechnol,* 1**,** 45-50.

PAUL, J. H., SULLIVAN, M. B., SEGALL, A. M. & ROHWER, F. 2002. Marine phage genomics. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 133**,** 463-476.

PEARSON, J. P., GRAY, K. M., PASSADOR, L., TUCKER, K. D., EBERHARD, A., IGLEWSKI, B. H., et al. 1994. Structure of the autoinducer required for expression of Pseudomonas aeruginosa virulence genes. *Proceedings of the National Academy of Sciences,* 91**,** 197-201.

PEARSON, J. P., PASSADOR, L., IGLEWSKI, B. H. & GREENBERG, E. 1995. A second N-acylhomoserine lactone signal produced by Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences,* 92**,** 1490-1494.

PEDULLA, M. L., FORD, M. E., HOUTZ, J. M., KARTHIKEYAN, T., WADSWORTH, C., LEWIS, J. A., et al. 2003a. Origins of highly mosaic mycobacteriophage genomes. *Cell,* 113**,** 171-182.

PEDULLA, M. L., FORD, M. E., HOUTZ, J. M., KARTHIKEYAN, T., WADSWORTH, C., LEWIS, J. A., et al. 2003b. Origins of Highly Mosaic Mycobacteriophage Genomes. *Cell,* 113**,** 171-182.

PEETERS, E., NELIS, H. J. & COENYE, T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of Microbiological Methods,* 72**,** 157-165.

PELOUX, Y. 1985. [Opportunistic bacteria]. *Ann Biol Clin (Paris),* 43**,** 153-5.

PEMBERTON, J. 1973. F116: a DNA bacteriophage specific for the pili of Pseudomonas aeruginosa strain PAO. *Virology,* 55**,** 558-560.

PERNA, N. T., PLUNKETT, G., BURLAND, V., MAU, B., GLASNER, J. D., ROSE, D. J., et al. 2001. Genome sequence of enterohaemorrhagic Escherichia coli O157: H7. *Nature,* 409**,** 529-533.

PERSSON, M., TARS, K. & LILJAS, L. 2008. The capsid of the small RNA phage PRR1 is stabilized by metal ions. *Journal of molecular biology,* 383**,** 914-922.

PESCI, E. C., MILBANK, J. B., PEARSON, J. P., MCKNIGHT, S., KENDE, A. S., GREENBERG, E. P., et al. 1999. Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences,* 96**,** 11229-11234.

PESCI, E. C., PEARSON, J. P., SEED, P. C. & IGLEWSKI, B. H. 1997. Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. *Journal of bacteriology,* 179**,** 3127-3132.

PICARD, B., DENAMUR, E., BARAKAT, A., ELION, J. & GOULLET, P. 1994. Genetic heterogeneity of Pseudomonas aeruginosa clinical isolates revealed by esterase electrophoretic polymorphism and restriction fragment length polymorphism of the ribosomal RNA gene region. *Journal of medical microbiology,* 40**,** 313-322.

PIER, G. B. 2007. Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *Int J Med Microbiol,* 297**,** 277-95.

PILEWSKI, J. M. & FRIZZELL, R. A. 1999. Role of CFTR in airway disease. *Physiol Rev,* 79**,** S215-55.

PINGOUD, A. & JELTSCH, A. 2001. Structure and function of type II restriction endonucleases. *Nucleic acids research,* 29**,** 3705-3727.

POOLE, K. 2001. Multidrug efflux pumps and antimicrobial resistance in Pseudomonas aeruginosa and related organisms. *Journal of molecular microbiology and biotechnology,* 3**,** 255-264.

POOLE, K., KREBES, K., MCNALLY, C. & NESHAT, S. 1993. Multiple antibiotic resistance in Pseudomonas aeruginosa: evidence for involvement of an efflux operon. *Journal of bacteriology,* 175**,** 7363-7372.

PRESSLER, T., FREDERIKSEN, B., SKOV, M., GARRED, P., KOCH, C. & HØIBY, N. 2006. Early rise of anti-Pseudomonas antibodies and a mucoid phenotype of Pseudomonas aeruginosa are risk factors for development of chronic lung infection—A case control study. *Journal of Cystic Fibrosis,* 5**,** 9-15.

PRIBBLE, C. G., BLACK, P. G., BOSSO, J. A. & TURNER, R. B. 1990. Clinical manifestations of exacerbations of cystic fibrosis associated with nonbacterial infections. *The Journal of Pediatrics,* 117**,** 200-204.

PTASHNE, M. 1967. Isolation of the λ phage repressor. *Proceedings of the National Academy of Sciences of the United States of America,* 57**,** 306.

PTASHNE, M. 2004. *A genetic switch: phage lambda revisited*, Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY:.

PTASHNE, M. & SWITCH, A. G. 1992. Phage Lambda and Higher Organisms. *Cell and Blackwell Scientific, Cambridge, MA*.

PUJOL, M. & GUDIOL, F. 2001. Evidence for antibiotic cycling in control of resistance. *Curr Opin Infect Dis,* 14**,** 711-5.

QIU, X., GURKAR, A. U. & LORY, S. 2006. Interstrain transfer of the large pathogenicity island (PAPI-1) of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences,* 103**,** 19830-19835.

QUILES-PUCHALT, N., CARPENA, N., ALONSO, J. C., NOVICK, R. P., MARINA, A. & PENADÉS, J. R. 2014. Staphylococcal pathogenicity island DNA packaging system involving cos-site packaging and phage-encoded HNH endonucleases. *Proceedings of the National Academy of Sciences,* 111**,** 6016-6021.

QUINN, R. A., LIM, Y. W., MAUGHAN, H., CONRAD, D., ROHWER, F. & WHITESON, K. L. 2014. Biogeochemical Forces Shape the Composition and Physiology of Polymicrobial Communities in the Cystic Fibrosis Lung. *mBio,* 5**,** e00956-13.

QUIRÓS, P., COLOMER-LLUCH, M., MARTÍNEZ-CASTILLO, A., MIRÓ, E., ARGENTE, M., JOFRE, J., et al. 2014. Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. *Antimicrobial agents and chemotherapy,* 58**,** 606-609.

RAKHUBA, D., KOLOMIETS, E., DEY, E. S. & NOVIK, G. 2010. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Pol J Microbiol,* 59**,** 145-155.

RALEIGH, E. A. & WILSON, G. 1986. Escherichia coli K-12 restricts DNA containing 5-methylcytosine. *Proceedings of the National Academy of Sciences,* 83**,** 9070-9074.

RAMIREZ, M., SEVERINA, E. & TOMASZ, A. 1999. A High Incidence of Prophage Carriage among Natural Isolates of Streptococcus pneumoniae. *Journal of Bacteriology,* 181**,** 3618-3625.

RAMSEY, B. W., DORKIN, H. L., EISENBERG, J. D., GIBSON, R. L., HARWOOD, I. R., KRAVITZ, R. M., et al. 1993. Efficacy of aerosolized tobramycin in patients with cystic fibrosis. *New England Journal of Medicine,* 328**,** 1740-1746.

RAMSEY, B. W., GORE, E. J., SMITH, A. L., COONEY, M. K., REDDING, G. J. & FOY, H. 1989. The effect of respiratory viral infections on patients with cystic fibrosis. *American journal of diseases of children,* 143**,** 662-668.

RANDALL-HAZELBAUER, L. & SCHWARTZ, M. 1973. Isolation of the bacteriophage lambda receptor from Escherichia coli. *Journal of bacteriology,* 116**,** 1436-1446.

READING, C. & COLE, M. 1977. Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from Streptomyces clavuligerus. *Antimicrobial Agents and Chemotherapy,* 11**,** 852-857.

REITER, W.-D., PALM, P. & YEATS, S. 1989. Transfer RNA genes frequently serve as integration sites for prokaryotic genetic elements. *Nucleic acids research,* 17**,** 1907-1914.

RENDELL, N. B., TAYLOR, G. W., SOMERVILLE, M., TODD, H., WILSON, R. & COLE, P. J. 1990. Characterisation of Pseudomonas rhamnolipids. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism,* 1045**,** 189-193.

REO, N. V. 2002. NMR-based metabolomics. *Drug Chem Toxicol,* 25**,** 375-82.

RIESENFELD, C. S., SCHLOSS, P. D. & HANDELSMAN, J. 2004. Metagenomics: genomic analysis of microbial communities. *Annu Rev Genet,* 38**,** 525-52.

RIORDAN, J. R., ROMMENS, J. M., KEREM, B.-S., ALON, N., ROZMAHEL, R., GRZELCZAK, Z., et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science,* 245**,** 1066-1073.

RITTER, A., BLUM, G., EMÖDY, L., KERENYI, M., BÖCK, A., NEUHIERI, B., et al. 1995. tRNA genes and pathogenicity islands: influence on virulence and metabolic properties of uropathogenic Escherichia coli. *Molecular microbiology,* 17**,** 109-121.

ROBERTS, R. C., STRÖM, A. R. & HELINSKI, D. R. 1994. The parDE operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. *Journal of Molecular Biology,* 237**,** 35-51.

ROBERTS, R. J., VINCZE, T., POSFAI, J. & MACELIS, D. 2010. REBASE--a database for DNA restriction and modification: enzymes, genes and genomes. *Nucleic Acids Res,* 38**,** D234-6.

RODRIGUEZ-BRITO, B., LI, L., WEGLEY, L., FURLAN, M., ANGLY, F., BREITBART, M., et al. 2010. Viral and microbial community dynamics in four aquatic environments. *The ISME journal,* 4**,** 739-751.

RODRÍGUEZ-GIRONÉS, M. A. & SANTAMARÍA, L. 2006. A new algorithm to calculate the nestedness temperature of presence–absence matrices. *Journal of Biogeography,* 33**,** 924-935.

ROEHM, N. W., RODGERS, G. H., HATFIELD, S. M. & GLASEBROOK, A. L. 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *Journal of immunological methods,* 142**,** 257-265.

ROGERS, G. B., CARROLL, M. P., SERISIER, D. J., HOCKEY, P. M., JONES, G. & BRUCE, K. D. 2004. Characterization of Bacterial Community Diversity in Cystic Fibrosis Lung Infections by Use of 16S Ribosomal DNA Terminal Restriction Fragment Length Polymorphism Profiling. *Journal of Clinical Microbiology,* 42**,** 5176-5183.

ROGERS, G. B., VAN DER GAST, C. J., CUTHBERTSON, L., THOMSON, S. K., BRUCE, K. D., MARTIN, M. L., et al. 2013. Clinical measures of disease in adult non-CF bronchiectasis correlate with airway microbiota composition. *Thorax,* 68**,** 731-737.

ROHWER, F., SEGALL, A., STEWARD, G., SEGURITAN, V., BREITBART, M., WOLVEN, F., et al. 2000. The complete genomic sequence of the marine phage Roseophage SIO1 shares homology with nonmarine phages. *Limnology and Oceanography,* 45**,** 408-418.

RÖMLING, U., KADER, A., SRIRAMULU, D. D., SIMM, R. & KRONVALL, G. 2005. Worldwide distribution of Pseudomonas aeruginosa clone C strains in the

aquatic environment and cystic fibrosis patients. *Environmental microbiology,* 7**,** 1029-1038.

RONCERO, C., DARZINS, A. & CASADABAN, M. 1990. Pseudomonas aeruginosa transposable bacteriophages D3112 and B3 require pili and surface growth for adsorption. *Journal of bacteriology,* 172**,** 1899-1904.

RONDON, M. R., AUGUST, P. R., BETTERMANN, A. D., BRADY, S. F., GROSSMAN, T. H., LILES, M. R., et al. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl Environ Microbiol,* 66**,** 2541-7.

ROOKS, D. J., YAN, Y., MCDONALD, J. E., WOODWARD, M. J., MCCARTHY, A. J. & ALLISON, H. E. 2010. Development and validation of a qPCR-based method for quantifying Shiga toxin-encoding and other lambdoid bacteriophages. *Environmental microbiology,* 12**,** 1194-204.

ROSVALL, M., DODD, I. B., KRISHNA, S. & SNEPPEN, K. 2006. Network models of phage-bacteria coevolution. *Physical Review E,* 74**,** 066105.

ROUM, J. H., BUHL, R., MCELVANEY, N. G., BOROK, Z. & CRYSTAL, R. G. 1993. Systemic deficiency of glutathione in cystic fibrosis. *J Appl Physiol (1985),* 75**,** 2419-24.

RUBIN, B. K., RAMIREZ, O., ZAYAS, J. G., FINEGAN, B. & KING, M. 1990. Collection and analysis of respiratory mucus from subjects without lung disease. *Am Rev Respir Dis,* 141**,** 1040-3.

RUSHTON, L., SASS, A., BALDWIN, A., DOWSON, C. G., DONOGHUE, D. & MAHENTHIRALINGAM, E. 2013. Key Role for Efflux in the Preservative Susceptibility and Adaptive Resistance of Burkholderia cepacia Complex Bacteria. *Antimicrobial Agents and Chemotherapy,* 57**,** 2972-2980.

RUSKA, H. 1941. Über ein neues bei der bakteriophagen Lyse auftretendes Formelement. *Naturwissenschaften,* 29**,** 367-368.

RYAN, A., KAPLAN, E., LAURIERI, N., LOWE, E. & SIM, E. 2011. Activation of nitrofurazone by azoreductases: multiple activities in one enzyme. *Scientific Reports,* 1**,** 63.

SAKO, T. & TSUCHIDA, N. 1983. Nucleotide sequence of the staphylokinase gene from Staphylococcus aurens. *Nucleic acids research,* 11**,** 7679-7693.

SALMEN, P., DWYER, D. M., VORSE, H. & KRUSE, W. 1983. WHirlpool-associated pseudomonas aeruginosa urinary tract infections. *JAMA,* 250**,** 2025-2026.

SALUNKHE, P., SMART, C. H., MORGAN, J. A. W., PANAGEA, S., WALSHAW, M. J., HART, C. A., et al. 2005. A cystic fibrosis epidemic strain of Pseudomonas aeruginosa displays enhanced virulence and antimicrobial resistance. *Journal of bacteriology,* 187**,** 4908-4920.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. 1989. *Molecular cloning: a laboratory manual,* New York, Cold Spring Harbor Laboratory.

SAMSON, J. E., MAGADAN, A. H., SABRI, M. & MOINEAU, S. 2013. Revenge of the phages: defeating bacterial defences. *Nat Rev Micro,* 11**,** 675-687.

SAMSYGINA, G. & BONI, E. 1984. [Bacteriophages and phage therapy in pediatric practice]. *Pediatriia***,** 67-70.

SANDERS, M. E. & KLAENHAMMER, T. R. 1983. Characterization of phage-sensitive mutants from a phage-insensitive strain of Streptococcus lactis: evidence for a plasmid determinant that prevents phage adsorption. *Applied and environmental microbiology,* 46**,** 1125-1133.

SANGER, F. 1975. The Croonian Lecture, 1975. Nucleotide sequences in DNA. *Proc R Soc Lond B Biol Sci,* 191**,** 317-33.

SANGER, F. & COULSON, A. R. 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology,* 94**,** 441-448.

SANGER, F., COULSON, A. R., HONG, G. F., HILL, D. F. & PETERSEN, G. B. 1982. Nucleotide sequence of bacteriophage λ DNA. *Journal of Molecular Biology,* 162**,** 729-773.

SASTRY, P., FINLAY, B. B., PASLOSKE, B., PARANCHYCH, W., PEARLSTONE, J. & SMILLIE, L. 1985. Comparative studies of the amino acid and nucleotide sequences of pilin derived from Pseudomonas aeruginosa PAK and PAO. *Journal of bacteriology,* 164**,** 571-577.

SAUNDERS, R. V., MODHA, D. E., CLAYDON, A. & GAILLARD, E. A. 2016. Chronic Aspergillus fumigatus colonization of the pediatric cystic fibrosis airway is common and may be associated with a more rapid decline in lung function. *Medical Mycology*.

SBERRO, H., LEAVITT, A., KIRO, R., KOH, E., PELEG, Y., QIMRON, U., et al. 2013. Discovery of Functional Toxin/Antitoxin Systems in Bacteria by Shotgun Cloning. *Molecular Cell,* 50**,** 136-148.

SCHAFFER, W. M. & ROSENZWEIG, M. L. 1978. Homage to the Red Queen. I. Coevolution of predators and their victims. *Theoretical Population Biology,* 14**,** 135-157.

SCHEMBRI, M. A., KJÆRGAARD, K. & KLEMM, P. 2003. Global gene expression in Escherichia coli biofilms. *Molecular microbiology,* 48**,** 253-267.

SCHLEHECK, D., BARRAUD, N., KLEBENSBERGER, J., WEBB, J. S., MCDOUGALD, D., RICE, S. A., et al. 2009. <italic>Pseudomonas aeruginosa</italic> PAO1 Preferentially Grows as Aggregates in Liquid Batch Cultures and Disperses upon Starvation. *PLoS ONE,* 4**,** e5513.

SCHMIDT, T. M., DELONG, E. & PACE, N. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *Journal of bacteriology,* 173**,** 4371-4378.

SCHOENFELD, T., PATTERSON, M., RICHARDSON, P. M., WOMMACK, K. E., YOUNG, M. & MEAD, D. 2008. Assembly of viral metagenomes from yellowstone hot springs. *Applied and environmental microbiology,* 74**,** 4164-4174.

SCHRECK, R., RIEBER, P. & BAEUERLE, P. A. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *Embo j,* 10**,** 2247-58.

SCHUCH, R. & FISCHETTI, V. A. 2009. The secret life of the anthrax agent Bacillus anthracis: bacteriophage-mediated ecological adaptations. *PLoS One,* 4**,** e6532.

SCHUERGERS, N. & WILDE, A. 2015. Appendages of the cyanobacterial cell. *Life,* 5**,** 700-715.

SCHWARTZ, M. & ROSSOFF, L. 1994. Tracheobronchomegaly. *CHEST Journal,* 106**,** 1589-1590.

SCHWARZMANN, S. W., ADLER, J. L., SULLIVAN, R. J., JR & MARINE, W. M. 1971. Bacterial pneumonia during the hong kong influenza epidemic of 1968-1969: Experience in a city-county hospital. *Archives of Internal Medicine,* 127**,** 1037-1041.

SCOTT, F. W. & PITT, T. L. 2004. Identification and characterization of transmissible Pseudomonas aeruginosa strains in cystic fibrosis patients in England and Wales. *Journal of medical microbiology,* 53**,** 609-15.

SEE, H. & WARK, P. 2008. Innate immune response to viral infection of the lungs. *Paediatric respiratory reviews,* 9**,** 243-250.

SEMMLER, A. B., WHITCHURCH, C. B. & MATTICK, J. S. 1999. A re-examination of twitching motility in Pseudomonas aeruginosa. *Microbiology,* 145**,** 2863-2873.

SEN, C. K. & PACKER, L. 1996. Antioxidant and redox regulation of gene transcription. *Faseb j,* 10**,** 709-20.

SHEPPARD, D. N. & WELSH, M. J. 1999. Structure and function of the CFTR chloride channel. *Physiological Reviews,* 79**,** S23-S45.

SHERRARD, L. J., TUNNEY, M. M. & ELBORN, J. S. 2014. Antimicrobial resistance in the respiratory microbiota of people with cystic fibrosis. *The Lancet,* 384**,** 703-713.

SHLAES, D. M. 2013. New β-lactam–β-lactamase inhibitor combinations in clinical development. *Annals of the New York Academy of Sciences,* 1277**,** 105-114.

SHOCKCOR, J., NICHOLS, A., ANTTI, H., PLUMB, R., CASTRO-PEREZ, J., MAJOR, H., et al. LC-MS/MS approach to'metabonomics'-What can it do for drug discovery/development? DRUG METABOLISM REVIEWS, 2003. MARCEL DEKKER INC 270 MADISON AVE, NEW YORK, NY 10016 USA, 1-1.

SHOTLAND, Y., KOBY, S., TEFF, D., MANSUR, N., OREN, D. A., TATEMATSU, K., et al. 1997. Proteolysis of the phage lambda CII regulatory protein by FtsH (HflB) of Escherichia coli. *Mol Microbiol,* 24**,** 1303-10.

SHOTLAND, Y., SHIFRIN, A., ZIV, T., TEFF, D., KOBY, S., KOBILER, O., et al. 2000. Proteolysis of bacteriophage lambda CII by Escherichia coli FtsH (HflB). *J Bacteriol,* 182**,** 3111-6.

SIBLEY, C. D., GRINWIS, M. E., FIELD, T. R., ESHAGHURSHAN, C. S., FARIA, M. M., DOWD, S. E., et al. 2011. Culture Enriched Molecular Profiling of the Cystic Fibrosis Airway Microbiome. *PLoS ONE,* 6**,** e22702.

SILBY, M. W., WINSTANLEY, C., GODFREY, S. A. C., LEVY, S. B. & JACKSON, R. W. 2011. Pseudomonas genomes: diverse and adaptable. *FEMS Microbiology Reviews,* 35**,** 652-680.

SIMMONDS, E., CONWAY, S., GHONEIM, A., ROSS, H. & LITTLEWOOD, J. 1990. Pseudomonas cepacia: a new pathogen in patients with cystic fibrosis referred to a large centre in the United Kingdom. *Archives of disease in childhood,* 65**,** 874-877.

SIMÕES, L. C., SIMÕES, M. & VIEIRA, M. J. 2007. Biofilm Interactions between Distinct Bacterial Genera Isolated from Drinking Water. *Applied and Environmental Microbiology,* 73**,** 6192-6200.

SIMON, M. N., DAVIS, R. W. & DAVIDSON, N. 1971. Heteroduplexes of DNA molecules of lambdoid phages: physical mapping of their base sequence relationships by electron microscopy. *Cold Spring Harbor Monograph Archive,* 2**,** 313-328.

SIMPSON, D. J., SACHER, J. C. & SZYMANSKI, C. M. 2015. Exploring the interactions between bacteriophage-encoded glycan binding proteins and carbohydrates. *Curr Opin Struct Biol,* 34**,** 69-77.

SINGANAYAGAM, A., JOSHI, P. V., MALLIA, P. & JOHNSTON, S. L. 2012. Viruses exacerbating chronic pulmonary disease: the role of immune modulation. *BMC Medicine,* 10**,** 1-12.

SINKUNAS, T., GASIUNAS, G., FREMAUX, C., BARRANGOU, R., HORVATH, P. & SIKSNYS, V. 2011. Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. *The EMBO journal,* 30**,** 1335-1342.

SKERKER, J. M. & BERG, H. C. 2001. Direct observation of extension and retraction of type IV pili. *Proceedings of the National Academy of Sciences,* 98**,** 6901-6904.

SKERKER, J. M. & SHAPIRO, L. 2000. Identification and cell cycle control of a novel pilus system in Caulobacter crescentus. *The EMBO Journal,* 19**,** 3223-3234.

SLACK, M. E. & NICHOLS, W. 1981. The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of Pseudomonas aeruginosa. *The Lancet,* 318**,** 502-503.

SMITH, C. B., GOLDEN, C., KLAUBER, M. R., KANNER, R. & RENZETTI, A. 1976. Interactions between viruses and bacteria in patients with chronic bronchitis. *J Infect Dis,* 134**,** 552-61.

SMITH, D., GUMERY, L., SMITH, E., STABLEFORTH, D., KAUFMANN, M. & PITT, T. 1993. Epidemic of Pseudomonas cepacia in an adult cystic fibrosis unit: evidence of person-to-person transmission. *Journal of clinical microbiology,* 31**,** 3017-3022.

SMITH, D. L., ROOKS, D. J., FOGG, P. C., DARBY, A. C., THOMSON, N. R., MCCARTHY, A. J., et al. 2012. Comparative genomics of Shiga toxin encoding bacteriophages. *BMC genomics,* 13**,** 311.

SMITH, D. L., WAREING, B. M., FOGG, P. C., RILEY, L. M., SPENCER, M., COX, M. J., et al. 2007. Multilocus characterization scheme for shiga toxin-encoding bacteriophages. *Applied and environmental microbiology,* 73**,** 8032-40.

SMITH, E. E., BUCKLEY, D. G., WU, Z., SAENPHIMMACHAK, C., HOFFMAN, L. R., D'ARGENIO, D. A., et al. 2006. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proceedings of the National Academy of Sciences,* 103**,** 8487-8492.

SMITH, H. O. & NATHANS, D. 1973. A Suggested nomenclature for bacterial host modification and restriction systems and their enzymes. *Journal of Molecular Biology,* 81**,** 419-423.

SMITH, H. O. & WELCOX, K. 1970. A restriction enzyme from Hemophilus influenzae: I. Purification and general properties. *Journal of molecular biology,* 51**,** 379-391.

SMITH, H. S., PIZER, L. I., PYLKAS, L. & LEDERBERG, S. 1969. Abortive infection of Shigella dysenteriae P2 by T2 bacteriophage. *Journal of virology,* 4**,** 162-168.

SMYTH, A., SMYTH, R., TONG, C., HART, C. & HEAF, D. 1995. Effect of respiratory virus infections including rhinovirus on clinical status in cystic fibrosis. *Archives of disease in childhood,* 73**,** 117-120.

SNYDER, L. R., KIRKLAND, J. J. & DOLAN, J. W. 2011. *Introduction to modern liquid chromatography*, John Wiley & Sons.

SOMMER, J. M. & NEWTON, A. 1988. Sequential regulation of developmental events during polar morphogenesis in Caulobacter crescentus: assembly of pili on swarmer cells requires cell separation. *Journal of Bacteriology,* 170**,** 409-415.

SONKAR, K., PURUSOTTAM, R. N. & SINHA, N. 2012. Metabonomic Study of Host–Phage Interaction by Nuclear Magnetic Resonance- and Statistical Total Correlation Spectroscopy-Based Analysis. *Analytical Chemistry,* 84**,** 4063-4070.

SONNENSCHEIN, C. 1927. Die Mucosus-form des Pyocyaneus-bakteriums, Bacterium pyocyaneum mucosum. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig,* 104**,** 365-373.

SPEERT, D., FARMER, S., CAMPBELL, M., MUSSER, J., SELANDER, R. & KUO, S. 1990. Conversion of Pseudomonas aeruginosa to the phenotype characteristic of strains from patients with cystic fibrosis. *Journal of clinical microbiology,* 28**,** 188-194.

SPINO, M., CHAI, R. P., ISLES, A. F., THIESSEN, J. J., TESORO, A., GOLD, R., et al. 1984. Cloxacillin absorption and disposition in cystic fibrosis. *The Journal of pediatrics,* 105**,** 829-835.

SPRATT, B. G. 2004. Exploring the concept of clonality in bacteria. *Genomics, Proteomics, and Clinical Bacteriology.* Springer.

STARKEY, M., HICKMAN, J. H., MA, L., ZHANG, N., DE LONG, S., HINZ, A., et al. 2009. Pseudomonas aeruginosa rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *Journal of bacteriology,* 191**,** 3492-3503.

STEFANIDES, M. M., COPELAND, C. E., KOMINOS, S. D. & YEE, R. B. 1976. In vitro penetration of topical antiseptics through eschar of burn patients. *Annals of Surgery,* 183**,** 358-364.

STEIN, D. C., GUNN, J. S., RADLINSKA, M. & PIEKAROWICZ, A. 1995. Restriction and modification systems of Neisseria gonorrhoeae. *Gene,* 157**,** 19-22.

STEIN, J. L., MARSH, T. L., WU, K. Y., SHIZUYA, H. & DELONG, E. F. 1996. Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. *Journal of bacteriology,* 178**,** 591-599.

STERN, A. & SOREK, R. 2011. The phage-host arms race: Shaping the evolution of microbes. *BioEssays,* 33**,** 43-51.

STEWART, R. M., WIEHLMANN, L., ASHELFORD, K. E., PRESTON, S. J., FRIMMERSDORF, E., CAMPBELL, B. J., et al. 2011. Genetic characterization indicates that a specific subpopulation of Pseudomonas aeruginosa is associated with keratitis infections. *Journal of clinical microbiology,* 49**,** 993-1003.

STOVER, C. K., PHAM, X. Q., ERWIN, A. L., MIZOGUCHI, S. D., WARRENER, P., HICKEY, M. J., et al. 2000. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature,* 406**,** 959-64.

STUDEMEISTER, A. E. & QUINN, J. P. 1988. Selective imipenem resistance in Pseudomonas aeruginosa associated with diminished outer membrane permeability. *Antimicrobial Agents and Chemotherapy,* 32**,** 1267-1268.

STURINO, J. M. & KLAENHAMMER, T. R. 2006. Engineered bacteriophage-defence systems in bioprocessing. *Nature Reviews Microbiology,* 4**,** 395-404.

SUAU, A., BONNET, R., SUTREN, M., GODON, J.-J., GIBSON, G. R., COLLINS, M. D., et al. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and environmental microbiology,* 65**,** 4799-4807.

SUGIYAMA, H. 1980. Clostridium botulinum neurotoxin. *Microbiological reviews,* 44**,** 419.

SULAKVELIDZE, A., ALAVIDZE, Z. & MORRIS, J. G. 2001. Bacteriophage therapy. *Antimicrobial agents and chemotherapy,* 45**,** 649-659.

SULLAM, P. M. 1994. Host-pathogen interactions in the development of bacterial endocarditis. *Current Opinion in Infectious Diseases,* 7**,** 304-309.

SUSSKIND, M. M., BOTSTEIN, D. & WRIGHT, A. 1974. Superinfection exclusion by P22 prophage in lysogens of Salmonella typhimurium: III. Failure of superinfecting phage DNA to enter sieA+ lysogens. *Virology,* 62**,** 350-366.

SUTHERLAND, I. W. 1999. Polysaccharases for microbial exopolysaccharides. *Carbohydrate Polymers,* 38**,** 319-328.

SUTHERLAND, I. W., HUGHES, K. A., SKILLMAN, L. C. & TAIT, K. 2004. The interaction of phage and biofilms. *FEMS Microbiology Letters,* 232**,** 1-6.

SUTTLE, C. A. 2005. Viruses in the sea. *Nature,* 437**,** 356-361.

SUTTLE, C. A. 2007. Marine viruses—major players in the global ecosystem. *Nature Reviews Microbiology,* 5**,** 801-812.

SVENSSON, O., KOURTI, T. & MACGREGOR, J. F. 2002. An investigation of orthogonal signal correction algorithms and their characteristics. *Journal of Chemometrics,* 16**,** 176-188.

SYRMIS, M. W., O'CARROLL, M. R., SLOOTS, T. P., COULTER, C., WAINWRIGHT, C. E., BELL, S. C., et al. 2004. Rapid genotyping of Pseudomonas aeruginosa isolates harboured by adult and paediatric patients with cystic fibrosis using repetitive-element-based PCR assays. *Journal of medical microbiology,* 53**,** 1089-1096.

TAKENOUCHI, T., SAKAGAWA, E. & SUGAWARA, M. 1999. Detection of gyrA Mutations among 335Pseudomonas aeruginosa Strains Isolated in Japan and Their Susceptibilities to Fluoroquinolones. *Antimicrobial agents and chemotherapy,* 43**,** 406-409.

TAKEYA, K. & AMAKO, K. 1966. A rod-shaped Pseudomonas phage. *Virology,* 28**,** 163-165.

TAMMA, P. D., COSGROVE, S. E. & MARAGAKIS, L. L. 2012. Combination Therapy for Treatment of Infections with Gram-Negative Bacteria. *Clinical Microbiology Reviews,* 25**,** 450-470.

TANEJA, S. K. & CHAKRAVORTY, M. 1978. Superinfection exclusion and changes in cellular transport processes in phage infected Salmonella typhimurium. *Molecular and General Genetics MGG,* 159**,** 293-296.

TANGALLAPALLY, R. P., YENDAPALLY, R., DANIELS, A. J., LEE, R. E. & LEE, R. E. 2007. Nitrofurans as novel anti-tuberculosis agents: identification, development and evaluation. *Curr Top Med Chem,* 7**,** 509-26.

TAPP, H. S. & KEMSLEY, E. K. 2009. Notes on the practical utility of OPLS. *TrAC Trends in Analytical Chemistry,* 28**,** 1322-1327.

TARIQ, M. A., EVEREST, F. L. C., COWLEY, L., DE SOYZA, A., HOLT, G. S., BRIDGE, S. H., et al. 2015. A Metagenomic approach to characterize temperate bacteriophage populations from cystic fibrosis and non-cystic fibrosis bronchiectasis patients. *Frontiers in Microbiology,* 6**,** 97.

TERNS, M. P. & TERNS, R. M. 2011. CRISPR-based adaptive immune systems. *Current Opinion in Microbiology,* 14**,** 321-327.

THEODORIDIS, G. A., GIKA, H. G., WANT, E. J. & WILSON, I. D. 2012. Liquid chromatography–mass spectrometry based global metabolite profiling: a review. *Analytica chimica acta,* 711**,** 7-16.

THOMASSEN, M., DEMKO, C., DOERSHUK, C., STERN, R. & KLINGER, J. 1986. Pseudomonas cepacia: decrease in colonization in patients with cystic fibrosis. *The American review of respiratory disease,* 134**,** 669-671.

THOMPSON, J. N. 1998. Rapid evolution as an ecological process. *Trends in Ecology & Evolution,* 13**,** 329-332.

TINGPEJ, P., SMITH, L., ROSE, B., ZHU, H., CONIBEAR, T., AL NASSAFI, K., et al. 2007. Phenotypic characterization of clonal and nonclonal Pseudomonas aeruginosa strains isolated from lungs of adults with cystic fibrosis. *Journal of clinical microbiology,* 45**,** 1697-1704.

TOCK, M. R. & DRYDEN, D. T. 2005. The biology of restriction and anti-restriction. *Curr Opin Microbiol,* 8**,** 466-72.

TODER, D. S., FERRELL, S. J., NEZEZON, J. L., RUST, L. & IGLEWSKI, B. H. 1994. lasA and lasB genes of Pseudomonas aeruginosa: analysis of transcription and gene product activity. *Infection and Immunity,* 62**,** 1320-1327.

TOMB, J. F., WHITE, O., KERLAVAGE, A. R., CLAYTON, R. A., SUTTON, G. G., FLEISCHMANN, R. D., et al. 1997. The complete genome sequence of the gastric pathogen Helicobacter pylori. *Nature,* 388**,** 539-47.

TOUW, D. 1998. Clinical pharmacokinetics of antimicrobial drugs in cystic fibrosis. *Pharmacy World and Science,* 20**,** 149-160.

TRAN, L. S. P., SZABÓ, L., PONYI, T., OROSZ, L., SÍK, T. & HOLCZINGER, A. 1999. Phage abortive infection of Bacillus licheniformis ATCC 9800; identification of the abiBL11 gene and localisation and sequencing of its promoter region. *Applied Microbiology and Biotechnology,* 52**,** 845-852.

TREMAROLI, V., WORKENTINE, M. L., WELJIE, A. M., VOGEL, H. J., CERI, H., VITI, C., et al. 2009. Metabolomic Investigation of the Bacterial Response to a Metal Challenge. *Applied and Environmental Microbiology,* 75**,** 719-728.

TÜMMLER, B. & KIEWITZ, C. 1999. Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. *Molecular medicine today,* 5**,** 351-358.

TUNNEY, M. M., FIELD, T. R., MORIARTY, T. F., PATRICK, S., DOERING, G., MUHLEBACH, M. S., et al. 2008. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *American journal of respiratory and critical care medicine,* 177**,** 995-1001.

TURNER, P., MCLENNAN, A., BATES, A. & WHITE, M. 2007. *BIOS Instant Notes in Molecular Biology*, Taylor & Francis.

TURTON, J. F., TURTON, S. E., YEARWOOD, L., YARDE, S., KAUFMANN, M. E. & PITT, T. L. 2010. Evaluation of a nine-locus variable-number tandem-repeat scheme for typing of Pseudomonas aeruginosa. *Clinical Microbiology and Infection,* 16**,** 1111-1116.

TWORT, F. W. 1915. An investigation on the nature of ultra-microscopic viruses. *The Lancet,* 186**,** 1241-1243.

UC-MASS, A., LOEZA, E. J., DE LA GARZA, M., GUARNEROS, G., HERNÁNDEZ-SÁNCHEZ, J. & KAMEYAMA, L. 2004. An orthologue of the cor gene is involved in the exclusion of temperate lambdoid phages. Evidence that Cor inactivates FhuA receptor functions. *Virology,* 329**,** 425-433.

VALLET, I., OLSON, J. W., LORY, S., LAZDUNSKI, A. & FILLOUX, A. 2001. The chaperone/usher pathways of Pseudomonas aeruginosa: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. *Proceedings of the National Academy of Sciences,* 98**,** 6911-6916.

VAN DER OOST, J., JORE, M. M., WESTRA, E. R., LUNDGREN, M. & BROUNS, S. J. 2009. CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends in biochemical sciences,* 34**,** 401-407.

VAN DER VLIET, A., O'NEILL, C. A., CROSS, C. E., KOOSTRA, J. M., VOLZ, W. G., HALLIWELL, B., et al. 1999. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol,* 276**,** L289-96.

VAN EWIJK, B. E., VAN DER ZALM, M. M., WOLFS, T. F. W. & VAN DER ENT, C. K. 2005. Viral respiratory infections in cystic fibrosis. *Journal of Cystic Fibrosis,* 4, Supplement 2**,** 31-36.

VAN HELVOORT, T. 1992. Bacteriological and physiological research styles in the early controversy on the nature of the bacteriophage phenomenon. *Medical history,* 36**,** 243-270.

VAN MELDEREN, L. & SAAVEDRA DE BAST, M. 2009. Bacterial Toxin–Antitoxin Systems: More Than Selfish Entities? *PLoS Genet,* 5**,** e1000437.

VAN MELDEREN, L., THI, M. H. D., LECCHI, P., GOTTESMAN, S., COUTURIER, M. & MAURIZI, M. R. 1996. ATP-dependent degradation of CcdA by Lon protease. Effects of secondary structure and heterologous subunit interactions. *Journal of Biological Chemistry,* 271**,** 27730-27738.

VAN VALEN, L. 1973. A new evolutionary law. Evol. Theory 1: 1—30. 1974. Two modes of evolution. *Nature,* 252**,** 298-299.

VARDAKAS, K. Z., TANSARLI, G. S., BLIZIOTIS, I. A. & FALAGAS, M. E. 2013. β-Lactam plus aminoglycoside or fluoroquinolone combination versus β-lactam monotherapy for Pseudomonas aeruginosa infections: A meta-analysis. *International Journal of Antimicrobial Agents,* 41**,** 301-310.

VASSEUR, P., VALLET-GELY, I., SOSCIA, C., GENIN, S. & FILLOUX, A. 2005. The pel genes of the Pseudomonas aeruginosa PAK strain are involved at early and late stages of biofilm formation. *Microbiology,* 151**,** 985-997.

VAUDAUX, P., KELLEY, W. L. & LEW, D. P. 2006. Staphylococcus aureus small colony variants: difficult to diagnose and difficult to treat. *Clinical infectious diseases,* 43**,** 968-970.

VERMIS, K., COENYE, T., MAHENTHIRALINGAM, E., NELIS, H. J. & VANDAMME, P. 2002. Evaluation of species-specific recA-based PCR tests for genomovar level identification within the Burkholderia cepacia complex. *Journal of medical microbiology,* 51**,** 937-940.

VERRON, T., SABATIER, R. & JOFFRE, R. 2004. Some theoretical properties of the O-PLS method. *Journal of Chemometrics,* 18**,** 62-68.

VICTORIA, J. G., KAPOOR, A., LI, L., BLINKOVA, O., SLIKAS, B., WANG, C., et al. 2009. Metagenomic Analyses of Viruses in Stool Samples from Children with Acute Flaccid Paralysis. *Journal of Virology,* 83**,** 4642-4651.

VILLAR, M. T., HELBER, J. T., HOOD, B., SCHAEFER, M. R. & HIRSCHBERG, R. L. 1999. Eikenella corrodens phase variation involves a posttranslational event in pilus formation. *Journal of bacteriology,* 181**,** 4154-4160.

VILLARREAL, J. V., JUNGFER, C., OBST, U. & SCHWARTZ, T. 2013. DNase I and Proteinase K eliminate DNA from injured or dead bacteria but not from living bacteria in microbial reference systems and natural drinking water biofilms for subsequent molecular biology analyses. *J Microbiol Methods,* 94**,** 161-9.

VIPRA, A., DESAI, S. N., JUNJAPPA, R. P., ROY, P., POONACHA, N., RAVINDER, P., et al. 2013. Determining the minimum inhibitory concentration of bacteriophages: potential advantages.

VOKES, S. A., REEVES, S. A., TORRES, A. G. & PAYNE, S. M. 1999. The aerobactin iron transport system genes in Shigella flexneri are present within a pathogenicity island. *Molecular microbiology,* 33**,** 63-73.

VON GOTZ, F., HAUSSLER, S., JORDAN, D., SARAVANAMUTHU, S. S., WEHMHONER, D., STRUSSMANN, A., et al. 2004. Expression analysis of a highly adherent and cytotoxic small colony variant of Pseudomonas aeruginosa isolated from a lung of a patient with cystic fibrosis. *J Bacteriol,* 186**,** 3837-47.

VOYNOW, J. A., GENDLER, S. J. & ROSE, M. C. 2006. Regulation of Mucin Genes in Chronic Inflammatory Airway Diseases. *American Journal of Respiratory Cell and Molecular Biology,* 34**,** 661-665.

WAGNER, P. L. & WALDOR, M. K. 2002. Bacteriophage control of bacterial virulence. *Infection and Immunity,* 70**,** 3985-3993.

WALDOR, M. K. & MEKALANOS, J. J. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science,* 272**,** 1910-4.

WANG, E. E., PROBER, C. G., MANSON, B., COREY, M. & LEVISON, H. 1984. Association of respiratory viral infections with pulmonary deterioration in patients with cystic fibrosis. *New England Journal of Medicine,* 311**,** 1653-1658.

WANG, G., BARTON, C. & RODGERS, F. G. 2002. Bacterial DNA decontamination for reverse transcription polymerase chain reaction (RT-PCR). *J Microbiol Methods,* 51**,** 119-21.

WANG, X., KIM, Y., MA, Q., HONG, S. H., POKUSAEVA, K., STURINO, J. M., et al. 2010. Cryptic prophages help bacteria cope with adverse environments. *Nature communications,* 1**,** 147.

WANG, X., LORD, D. M., CHENG, H.-Y., OSBOURNE, D. O., HONG, S. H., SANCHEZ-TORRES, V., et al. 2012. A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nature chemical biology,* 8**,** 855-861.

WANG, X. & WOOD, T. K. 2011. Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl Environ Microbiol,* 77**,** 5577-83.

WAT, D., GELDER, C., HIBBITTS, S., CAFFERTY, F., BOWLER, I., PIERREPOINT, M., et al. 2008. The role of respiratory viruses in cystic fibrosis. *Journal of Cystic Fibrosis,* 7**,** 320-328.

WATSON, P. R., GALYOV, E. E., PAULIN, S. M., JONES, P. W. & WALLIS, T. S. 1998. Mutation of invH, but Notstn, Reduces Salmonella-Induced Enteritis in Cattle. *Infection and Immunity,* 66**,** 1432-1438.

WEBB, J. S., LAU, M. & KJELLEBERG, S. 2004. Bacteriophage and phenotypic variation in Pseudomonas aeruginosa biofilm development. *Journal of bacteriology,* 186**,** 8066-73.

WECKWERTH, W. 2003. Metabolomics in systems biology. *Annual review of plant biology,* 54**,** 669-689.

WECKWERTH, W. & MORGENTHAL, K. 2005. Metabolomics: from pattern recognition to biological interpretation. *Drug discovery today,* 10**,** 1551-1558.

WEINBAUER, M. G. & RASSOULZADEGAN, F. 2004. Are viruses driving microbial diversification and diversity? *Environmental microbiology,* 6**,** 1-11.

WEISS, R. 1971. The structure and occurrence of pili (fimbriae) on Pseudomonas aeruginosa. *Journal of general microbiology,* 67**,** 135-144.

WEITZ, J. S., HARTMAN, H. & LEVIN, S. A. 2005. Coevolutionary arms races between bacteria and bacteriophage. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 9535-9540.

WELLINGHAUSEN, N., KÖTHE, J., WIRTHS, B., SIGGE, A. & POPPERT, S. 2005. Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical Pseudomonas aeruginosa, from patients with cystic fibrosis. *Journal of clinical microbiology,* 43**,** 4070-4075.

WESTBROCK-WADMAN, S., SHERMAN, D. R., HICKEY, M. J., COULTER, S. N., ZHU, Y. Q., WARRENER, P., et al. 1999. Characterization of a Pseudomonas aeruginosa efflux pump contributing to aminoglycoside impermeability. *Antimicrobial agents and chemotherapy,* 43**,** 2975-2983.

WESTMORELAND, B. C., SZYBALSKI, W. & RIS, H. 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. *Science,* 163**,** 1343-1348.

WHITCHURCH, C. B. & MATTICK, J. S. 1994. Characterization of a gene, pilU, required for twitching motility but not phage sensitivity in Pseudomonas aeruginosa. *Molecular microbiology,* 13**,** 1079-1091.

WHITE, A. J., GOMPERTZ, S. & STOCKLEY, R. A. 2003. Chronic obstructive pulmonary disease • 6: The aetiology of exacerbations of chronic obstructive pulmonary disease. *Thorax,* 58**,** 73-80.

WHITEHEAD, H. R. & COX, G. 1935. *The occurrence of bacteriophage in cultures of lactic streptococci: A preliminary note*, Government Printer.

WHITELEY, M., BANGERA, M. G., BUMGARNER, R. E., PARSEK, M. R., TEITZEL, G. M., LORY, S., et al. 2001. Gene expression in Pseudomonas aeruginosa biofilms. *Nature,* 413**,** 860-864.

WHO 2004. The molecular genetic epidemiology of cystic fibrosis: report of a joint meeting of WHO/ECFTN/ICF (M) A/ECFS. *Geneva, WHO*.

WHO. 2015. *Antibiotic Resistance: Fact sheet* [Online]. Available: http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/ [Accessed 02.02.16 2016].

WIEDENHEFT, B., LANDER, G. C., ZHOU, K., JORE, M. M., BROUNS, S. J., VAN DER OOST, J., et al. 2011. Structures of the RNA-guided surveillance complex from a bacterial immune system. *Nature,* 477**,** 486-489.

WILDER, C. N., ALLADA, G. & SCHUSTER, M. 2009. Instantaneous within-patient diversity of Pseudomonas aeruginosa quorum-sensing populations from cystic fibrosis lung infections. *Infect Immun,* 77**,** 5631-9.

WILLNER, D., FURLAN, M., HAYNES, M., SCHMIEDER, R., ANGLY, F. E. & SILVA, J. 2009. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PLoS One,* 4.

WILLNER, D., HAYNES, M. R., FURLAN, M., SCHMIEDER, R., LIM, Y. W., RAINEY, P. B., et al. 2012. Spatial distribution of microbial communities in the cystic fibrosis lung. *The ISME journal,* 6**,** 471-474.

WILSON, C. B., JONES, P. W., O'LEARY, C. J., COLE, P. J. & WILSON, R. 1997. Validation of the St. George's Respiratory Questionnaire in bronchiectasis. *American journal of respiratory and critical care medicine,* 156**,** 536-541.

WILSON, R. & COLE, P. J. 1988. The effect of bacterial products on ciliary function. *Am Rev Respir Dis,* 138**,** S49-53.

WINSTANLEY, C. & FOTHERGILL, J. L. 2009. The role of quorum sensing in chronic cystic fibrosis Pseudomonas aeruginosa infections. *FEMS microbiology letters,* 290**,** 1-9.

WINSTANLEY, C., KAYE, S. B., NEAL, T. J., CHILTON, H. J., MIKSCH, S. & HART, C. A. 2005. Genotypic and phenotypic characteristics of Pseudomonas aeruginosa isolates associated with ulcerative keratitis. *Journal of medical microbiology,* 54**,** 519-526.

WINSTANLEY, C., LANGILLE, M. G., FOTHERGILL, J. L., KUKAVICA-IBRULJ, I., PARADIS-BLEAU, C., SANSCHAGRIN, F., et al. 2009. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of Pseudomonas aeruginosa. *Genome research,* 19**,** 12-23.

WINZER, K., FALCONER, C., GARBER, N. C., DIGGLE, S. P., CAMARA, M. & WILLIAMS, P. 2000. The Pseudomonas aeruginosa lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS. *Journal of bacteriology,* 182**,** 6401-6411.

WISHART, D. S., JEWISON, T., GUO, A. C., WILSON, M., KNOX, C., LIU, Y., et al. 2013. HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res,* 41**,** D801-7.

WISHART, D. S., KNOX, C., GUO, A. C., EISNER, R., YOUNG, N., GAUTAM, B., et al. 2009. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res,* 37**,** D603-10.

WISHART, D. S., TZUR, D., KNOX, C., EISNER, R., GUO, A. C., YOUNG, N., et al. 2007. HMDB: the Human Metabolome Database. *Nucleic Acids Res,* 35**,** D521-6.

WOMMACK, K. E. & COLWELL, R. R. 2000. Virioplankton: viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews,* 64**,** 69-114.

WOO, J. K., WEBB, J. S., KIROV, S. M., KJELLEBERG, S. & RICE, S. A. 2012. Biofilm dispersal cells of a cystic fibrosis Pseudomonas aeruginosa isolate exhibit variability in functional traits likely to contribute to persistent infection. *FEMS Immunology & Medical Microbiology,* 66**,** 251-264.

WORKENTINE, M. L., SIBLEY, C. D., GLEZERSON, B., PURIGHALLA, S., NORGAARD-GRON, J. C., PARKINS, M. D., et al. 2013. Phenotypic Heterogeneity of Pseudomonas aeruginosa Populations in a Cystic Fibrosis Patient. *PLoS ONE,* 8**,** e60225.

WORLITZSCH, D., RINTELEN, C., BOHM, K., WOLLSCHLAGER, B., MERKEL, N., BORNEFF-LIPP, M., et al. 2009. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. *Clin Microbiol Infect,* 15**,** 454-60.

WORLITZSCH, D., TARRAN, R., ULRICH, M., SCHWAB, U., CEKICI, A., MEYER, K. C., et al. 2002. Effects of reduced mucus oxygen concentration in airway< i> Pseudomonas</i> infections of cystic fibrosis patients. *The Journal of clinical investigation,* 109**,** 317-325.

WRIGHT, E. A., FOTHERGILL, J. L., PATERSON, S., BROCKHURST, M. A. & WINSTANLEY, C. 2013. Sub-inhibitory concentrations of some antibiotics can drive diversification

of Pseudomonas aeruginosa populations in artificial sputum medium. *BMC Microbiology,* 13**,** 1-12.

YAFFE, S. J., GERBRACHT, L. M., MOSOVICH, L. L., MATTAR, M. E., DANISH, M. & JUSKO, W. J. 1977. Pharmacokinetics of methicillin in patients with cystic fibrosis. *Journal of Infectious Diseases,* 135**,** 828-831.

YANG, L., AMAD, M. A., WINNIK, W. M., SCHOEN, A. E., SCHWEINGRUBER, H., MYLCHREEST, I., et al. 2002. Investigation of an enhanced resolution triple quadrupole mass spectrometer for high-throughput liquid chromatography/tandem mass spectrometry assays. *Rapid Communications in Mass Spectrometry,* 16**,** 2060-2066.

ZELANTE, T., PIERACCINI, G., SCARINGI, L., AVERSA, F. & ROMANI, L. 2016. Learning from other diseases: protection and pathology in chronic fungal infections. *Seminars in Immunopathology,* 38**,** 239-248.

ZHANG, B. & POWERS, R. 2012. Analysis of bacterial biofilms using NMR-based metabolomics. *Future medicinal chemistry,* 4**,** 1273-1306.

ZHANG, W., LI, F. & NIE, L. 2010. Integrating multiple 'omics' analysis for microbial biology: application and methodologies. *Microbiology,* 156**,** 287-301.

ZIERDT, C. & SCHMIDT, P. 1964. Dissociation in Pseudomonas aeruginosa. *Journal of bacteriology,* 87**,** 1003-1010.

ZIERVOGEL, BRIGITTE K. & ROUX, B. 2013. The Binding of Antibiotics in OmpF Porin. *Structure,* 21**,** 76-87.

ZINDER, N. D. & LEDERBERG, J. 1952. Genetic exchange in Salmonella. *Journal of bacteriology,* 64**,** 679.

ZOBELL, J. T., WATERS, C. D., YOUNG, D. C., STOCKMANN, C., AMPOFO, K., SHERWIN, C. M., et al. 2013. Optimization of anti-pseudomonal antibiotics for cystic fibrosis pulmonary exacerbations: II. cephalosporins and penicillins. *Pediatric pulmonology,* 48**,** 107-122.